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Megalastrum **(Dryopteridaceae) in Brazil, Paraguay, and Uruguay**
Robbin C. Moran, Jefferson Prado, and Paulo H. Labiak **1**

Local Knowledge and Management of the Royal Fern (*Osmunda regalis* L.) in Northern Spain: Implications for Biodiversity Conservation
María Molina, Victoria Reyes-García, Manuel Pardo-de-Santayana **45**

Shorter Notes

Salvinia molesta **in Mexico** *Arturo Mora-Olivo and George Yatskievych* **56**

Type Specimens of *Dracoglossum sinuatum* Uncovered in the Rio de Janeiro Herbarium
Maarten J. M. Christenhusz **58**

Review

Illustrated Flora of Ferns and Fern Allies of South Pacific Islands
Barbara Joe Hoshizaki **59**

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Megalastrum (Dryopteridaceae) in Brazil, Paraguay, and Uruguay

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ABSTRACT.—We provide keys, descriptions, illustrations, full synonymies, maps, and a list of specimens examined for species of *Megalastrum* found in Brazil, Paraguay, and Uruguay. Eighteen species are recognized, including seven new ones: *M. albidum*, *M. brevipubens*, *M. indusiatum*, *M. littorale*, *M. organense*, *M. retrorsum*, and *M. substrigosum*. The species occur primarily in the coastal mountains; none occur in Amazonia. The mountains of coastal Brazil are a center of endemism and diversity for the genus.

KEY WORDS.—*Megalastrum*, Dryopteridaceae, Brazil, floristics, taxonomy, systematics, ferns

This paper treats the species of *Megalastrum* Holttum that occur in Brazil, Paraguay, and Uruguay. This region was chosen for treatment because, with a few exceptions, there is no overlap of species with other regions of the Neotropics. Most species of *Megalastrum* in the region treated here occur primarily in the Atlantic rainforest of coastal Brazil (*sensu* Rizzini, 1976). That there is hardly any overlap with other regions in the Neotropics is not surprising given that the Atlantic rainforest of coastal Brazil is isolated from the Andes, the main region where the genus occurs in South America. The genus is absent from Amazonian Brazil (Fig. 1).

Although Christensen (1913, 1920) treated 10 Brazilian species of *Megalastrum*, his treatment was based on the relatively few specimens then available and is now badly out-of-date. Brade (1972) published a helpful study of the Brazilian species, also including species now placed in *Dryopteris* and *Ctenitis*, but it too needs updating because recent collections have extended the known ranges of many Brazilian species and new species have been discovered. Moran *et al.* (2008) have shown that one of the species assigned to the group by Christensen, Brade, and others (*M. lasiernos* (Spreng.) A. R. Sm. & R. C. Moran) is actually a *Cyathea*.

Christensen (1913, 1920) first recognized *Megalastrum* as a group, treating them in his monograph of Neotropical *Dryopteris* as the "group of *D. subincisa*" of subgen. *Ctenitis*. He assigned about 30 species to the group. Holttum (1986) elevated the subincisa group to generic rank, as *Megalastrum*, and made combinations for the Jamaican type of the genus (*M. villosum* (L.)

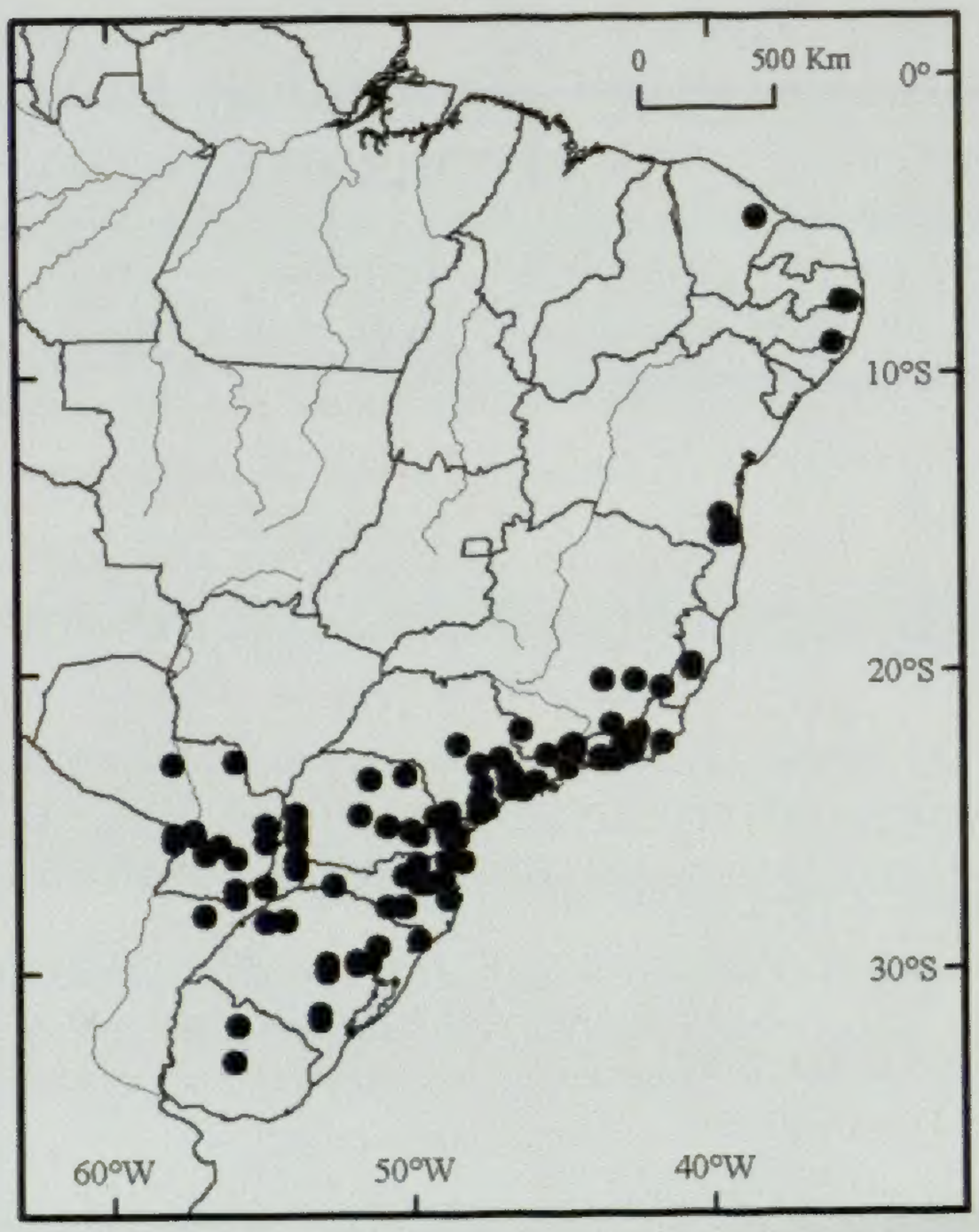

FIG. 1. Distribution of *Megalastrum* in Brazil, Paraguay, and Uruguay.

Holttum) and the African and Madagascan species. Combinations for 39 Neotropical species were subsequently made by Smith and Moran (1987). Since then, two new species have been described from Costa Rica (Rojas-Alvarado, 2001), one from Peru (Smith, 2006), and six from Bolivia (Kessler and Smith, 2006).

Megalastrum differs from other dryopteroid genera by lamina cutting, venation, and type of hairs on the axes adaxially. As one goes distally along the pinna, the basal basiscopic pinnules gradually become decurrent and broadly adnate to the pinna rachis. Correspondingly, the vein that supplies the broadly adnate segment or lobe arises from the costa, not the costule. This is unique among dryopteroid ferns.

Another helpful characteristic is the form of the hairs on the adaxial surfaces of pinna rachises. Generally, these are coarse, whitish, septate, sharp-tipped, and antrorsely strigose or spreading (Smith and Moran, 1987). Often they remain terete after drying, the cells not collapsing and twisting at right angles to each other.

The veins in most species of *Megalastrum* end before the lamina margins in enlarged clavate tips (hydathodes). This type of vein termination is uncommon among dryopteroid ferns, which typically have the vein tips slender and reaching the margin. The dryopteroid genera that have hydathodes are *Didymochlaena* (pers. obs.), *Elaphoglossum* sect. *Setosa* (Mickel and Atehortúa, 1980; Moran *et al.*, 2007), and *Stigmatopteris* (Moran, 1991). Judging from the dryopteroid portion of the cladogram presented by Schuettpelz and Pryer

(2007), the presence of hydathodes in these genera represents independent evolutionary developments.

The spores of *Megalastrum* are of two types: 1) densely echinate, and 2) cristate. The echinate type resembles that found occasionally in other dryopteroid ferns (e.g., *Elaphoglossum papillosum*, *E. pygmaeum*, *E. oblanceolatum*; Moran *et al.*, 2007). The cristate type, however, seems unique among dryopteroid ferns. Most dryopteroid ferns have broadly folded perispores, but the cristate spores of *Megalastrum* have sharp thin crests, and they are often parallel (see images at www.plantsystematics.org).

Previously, *Megalastrum* was thought to be closely related to *Ctenitis*, which it resembles in lamina cutting (Christensen, 1920). Recent DNA-based phylogenetic studies, however, reveal that *Rumohra* is the sister genus to *Megalastrum*, and that these two genera are sister to *Lastreopsis* (Schuettpelz and Pryer, 2007). This clade is sister to the "former lomariopsids" (*Bolbitis*, *Elaphoglossum*, *Lomagramma*, and *Teratophyllum*).

Megalastrum comprises medium- to large-sized ferns, often with finely divided laminae. This imparts challenges to working with herbarium specimens, which are often fragmentary, consisting only of a few pinnae or an apex. In decompound ferns, the laminae will vary from less divided juvenile leaves to highly divided large ones. Thus, two species that differ greatly in the division of their laminae might appear to overlap in keys and descriptions, even though their dissection is, on the whole, quite different. For this reason we give in the descriptions the cutting for the basal pinnae, where the laminae are most finely divided.

Given the problems with using lamina division, we emphasize the more consistent characteristics of the hairs and scales in our keys and comparative discussion. Hairs and scales in *Megalastrum* are readily distinct. The hairs are often whitish, uniseriate, and multicellular, whereas scales are usually broad, and flat or bullate, with reddish walls and denticulate margins. Towards the lamina margins, the scales become gradually reduced to uniseriate structures that resemble hairs. If these are interpreted as hairs, it will cause confusion in using the keys and descriptions. These reduced hairs, or "proscales," are called uniseriate scales in this paper. They are usually appressed, reddish, and several-celled. That they are reduced scales is shown by their complete transition with large, broader scales; i.e., proscales are serially homologous with typical scales.

In some species, the hairs are modified as glands. The glandular cell is spherical and typically yellowish (less commonly reddish). Glandular hairs may be capitate, that is, each with a glandular cell at its apex (Fig. 10R, W), or they may be sessile (Fig. 10E). The glands are only 0.05–0.1 mm long and are best seen with at least 30 times magnification.

Megalastrum is mostly Neotropical, occurring from Mexico and Cuba to southern Chile. Three species occur in the Old World (Africa, Comores, Réunion, Madagascar). Brazil shares four species with Paraguay and Uruguay (Table 1).

TABLE 1. Distribution of the *Megalastrum* in Brazil, Paraguay, and Uruguay.

Brazil
Alagoas (1): *eugenii*
Bahia (6): *canescens, connexum, eugenii, grande, indusiatum, umbrinum*
Ceará (1): *eugenii*
Espírito Santo (4): *connexum, grande, substrigosum, wacketii*
Minas Gerais (4): *abundans, connexum, crenulans, umbrinum*
Paraná (6): *abundans, albidum, brevipubens, connexum, crenulans, umbrinum*
Pernambuco (1): *eugenii*
Rio de Janeiro (10): *abundans, canescens, connexum, crenulans, grande, inaequale, littorale, organense, retrorsum, umbrinum*
Rio Grande do Sul (6): *abundans, adenopteris, connexum, crenulans, oreocharis, umbrinum*
Santa Catarina (5): *abundans, adenopteris, connexum, oreocharis, umbrinum*
São Paulo (11): *albidum, brevipubens, canescens, connexum, crenulans, grande, inaequale, littorale, organense, umbrinum, wacketii*

Paraguay
Alto Paraná (1): *connexum*
Amambay (2): *connexum, umbrinum*
Caaguazú (1): *connexum*
Caazapa (1): *connexum*
Central (2): *connexum, crenulans*
Guaira (1): *brevipubens*
Paraguarí (1): *connexum*
San Bernardino (1): *connexum*

Uruguay
Rivera (1): *connexum*
Tucuarembó (2): *connexum, oreocharis*

METHODS

Herbarium specimens were borrowed from 23 herbaria (see Acknowledgements). Living plants were studied in the field by two of us (Prado and Labiak). To show the variation in lamina cutting, silhouettes were prepared from herbarium specimens for all species. Digital images were taken of basal pinna, and the images were then adjusted to provide a white background and a black lamina. To produce the distribution maps, the geographic coordinates were estimated for many specimens because this information was not provided on the labels. Our estimates of geographic coordinates are given in brackets in the Specimens Examined section below. For the Specimens Examined, only two or three specimens were cited per state. The dot distribution maps based on all specimens were generated in the GIS laboratory at the New York Botanical Garden.

RESULTS

Geography.—Two-thirds of the species of *Megalastrum* treated here are restricted to the coastal mountains of southeastern Brazil (Table 1). Four of these species (*M. littorale*, *M. organense*, *M. retrorsum*, and *M. wacketii*) are narrow endemics, occurring collectively only in the mountains in the states of

São Paulo and Rio de Janeiro. Species that occur beyond the coastal mountains are *M. adenopteris*, *M. brevipubens*, *M. connexum*, *M. crenulans*, *M. oreocharis*, and *M. umbrinum*. Of these, *M. connexum* has the widest distribution, occurring from Bahia, Brazil, to Tucarembó, Uruguay. Given these distributions, the coastal mountains of Brazil are a center of endemism and diversity for the genus (Fig. 1).

Taxonomic treatment.—Eighteen species are here recognized for the region, seven of which are new. No infraspecific taxa are recognized. The species may be separated by the key below.

Megalastrum Holttum, Gard. Bull. Straits Settlem., ser. 3, 39: 161. 1986. TYPE.—*Megalastrum villosum* (L.) Holttum [basionym: *Polypodium villosum* L.].

Ctenitis subsect. *Subincisae* (C. Chr.) Tindale, Contr. New South Wales Natl. Herb. 3: 252. 1965. *Dryopteris* sect. *Subincisae* C. Chr., Index Filic., Suppl. 3: 7. 1934.

Plants terrestrial; rhizomes erect to decumbent; petioles scaly toward the base, with 4–10 vascular bundles, the two adaxial bundles enlarged; laminae 1-pinnate-pinnatifid to 4-pinnate-pinnatifid, catadromic above the basal pinnae; basal pinnae inequilateral and more developed on the basiscopic side or (less commonly) equilateral; rachises, costae, and costules not grooved or only shallowly so adaxially, scaly and pubescent abaxially, densely pubescent on the adaxial surfaces, the hairs whitish, spreading to antrorsely strigose, multicellular, if glands present, these ca. 0.1 mm wide, spherical, shiny, yellowish to orangish, sessile to stalked; basal basiscopic segment of more distal pinnules becoming decurrent and adnate to the pinna rachises, the vein supplying the segment springing from the pinna rachis instead of the costule; hydathodes (enlarged vein ends) present adaxially; indusia absent or (less commonly) present, circular, brown, firm, in some species minute and fugacious; $x=41$.

KEY TO THE SPECIES OF *MEGALASTRUM* IN BRAZIL, PARAGUAY, AND URUGUAY

1. Indusia present, persistent
   2. Indusia completely covering the sori; scales bullate on the pinna rachises abaxially; lamina tissue between the veins adaxially glandular . . . . . **7. M. crenulans**
   2. Indusia partially covering the sori, about the size of a single sporangium capsule; scales non-bullate on the pinna rachises abaxially; lamina tissue between the veins adaxially glabrous . . . . . **11. M. indusiatum**
1. Indusia absent or (in *M. adenopteris*) minute and fugacious
   3. Laminae glandular abaxially
      4. Hairs on the abaxial surfaces ca. 1–2 mm long, 2–8-celled; glands on the lamina tissue abaxially stalked, never sessile
         5. Laminae 2-pinnate-pinnatisect at base, 2-pinnate-pinnatifid medially; petioles and rachises glabrescent . . . . . **5. M. canescens**
         5. Laminae 3-pinnate-pinnatisect at base, 3-pinnate medially; petioles and rachises conspicuously pubescent. . . . . **12. M. littorale**

4. Hairs on the abaxial surfaces ca. 0.1–0.2 mm long, 1- or 2-celled; glands on the lamina tissue abaxially sessile or short-stalked
6. Hairs on the abaxial surfaces of the costules more than 1 mm long . . . **18. M. wacketii**
6. Hairs on the abaxial surfaces of the costules 0.2–0.3 mm long
7. Laminae adaxially densely and evenly pubescent between veins; rachis scales appressed, inconspicuous; minute fugacious indusia present . . . . **2. M. adenopteris**
7. Laminae adaxially glabrous between veins or sparsely pubescent with a few hairs near the margins; rachis scales spreading, conspicuous; minute fugacious indusia absent . . . . . **17. M. umbrinum**

3. Laminae eglandular abaxially
8. Scales of the petioles and rachises retrorsely denticulate
9. Lamina tissue between the veins pubescent on both surfaces . . . . . **15. M. retrorsum**
9. Laminar tissue between the veins glabrous on both surfaces . . . . . **14. M. organense**
8. Scales of the petioles and rachises entire, or if denticulate, not retrorsely so
10. Lamina tissue pubescent between the veins abaxially, the hairs erect or spreading, often acicular
11. Hairs on the abaxial surfaces of the lamina between the veins 0.4–0.6 mm long . . . . . **3. M. albidum**
11. Hairs on the abaxial surfaces of the lamina between the veins ca. 0.1 mm . . . . . **4. M. brevipubens**
10. Lamina tissue glabrous between the veins abaxially (sometimes with appressed reddish uniseriate scales, but no hairs)
12. Scales on the abaxial surfaces of the costae and costules sub-bullate to bullate
13. Laminae 3-pinnate-pinnatifid at base, subcoriaceous, lustrous adaxially . . . . . **10. M. inaequale**
13. Laminae 4-pinnate-pinnatifid at base, chartaceous, dull adaxially . . . . . **1. M. abundans**
12. Scales on the abaxial surfaces of the costae and costules flat (non-bullate)
14. Costae glabrous adaxially . . . . . **9. M. grande**
14. Costae pubescent adaxially
15. Hairs on the pinna rachises, costules, and veins abaxially 0.5–0.7 mm long . . . . . **13. M. oreocharis**
15. Hairs on the pinna rachises, costules, and veins abaxially 0.1–0.3 mm long
16. Pinna rachises abaxially densely pubescent, hairs substrigose . . . . . **16. M. substrigosum**
16. Pinna rachises abaxially glabrous or glabrescent, the hairs (when present) not substrigose
17. Laminae 1-pinnate-pinnatisect at the middle; petiole scales dark brown . . . . . **8. M. eugenii**
17. Laminae 2-pinnate-pinnatifid or more divided at the middle; petiole scales yellowish brown . . . . . **6. M. connexum**

1. ***Megalastrum abundans*** (Rosenst.) A. R. Sm. & R. C. Moran, Amer. Fern J. 77: 127. 1987. *Dryopteris abundans* Rosenst., Hedwigia 46: 133. 1906. *Ctenitis abundans* (Rosenst.) Copel., Ann. Crypt. Phytopath. [Gen. Fil.] 5: 124. 1947. LECTOTYPE (here designated).—BRAZIL. **Rio Grande do Sul**: Mun. Santa Cruz, [29°43′S, 52°52′W], Sette Lagoas do Herval do Paredão, 2 Mar 1905, *Jürgens 195* [Rosenstock Filices Austrobrasilienses no. 367] (NY; duplicates: B, NY, P, R-n.v., S-n.v.). **Figs. 2A, 5A, 8A–H.**

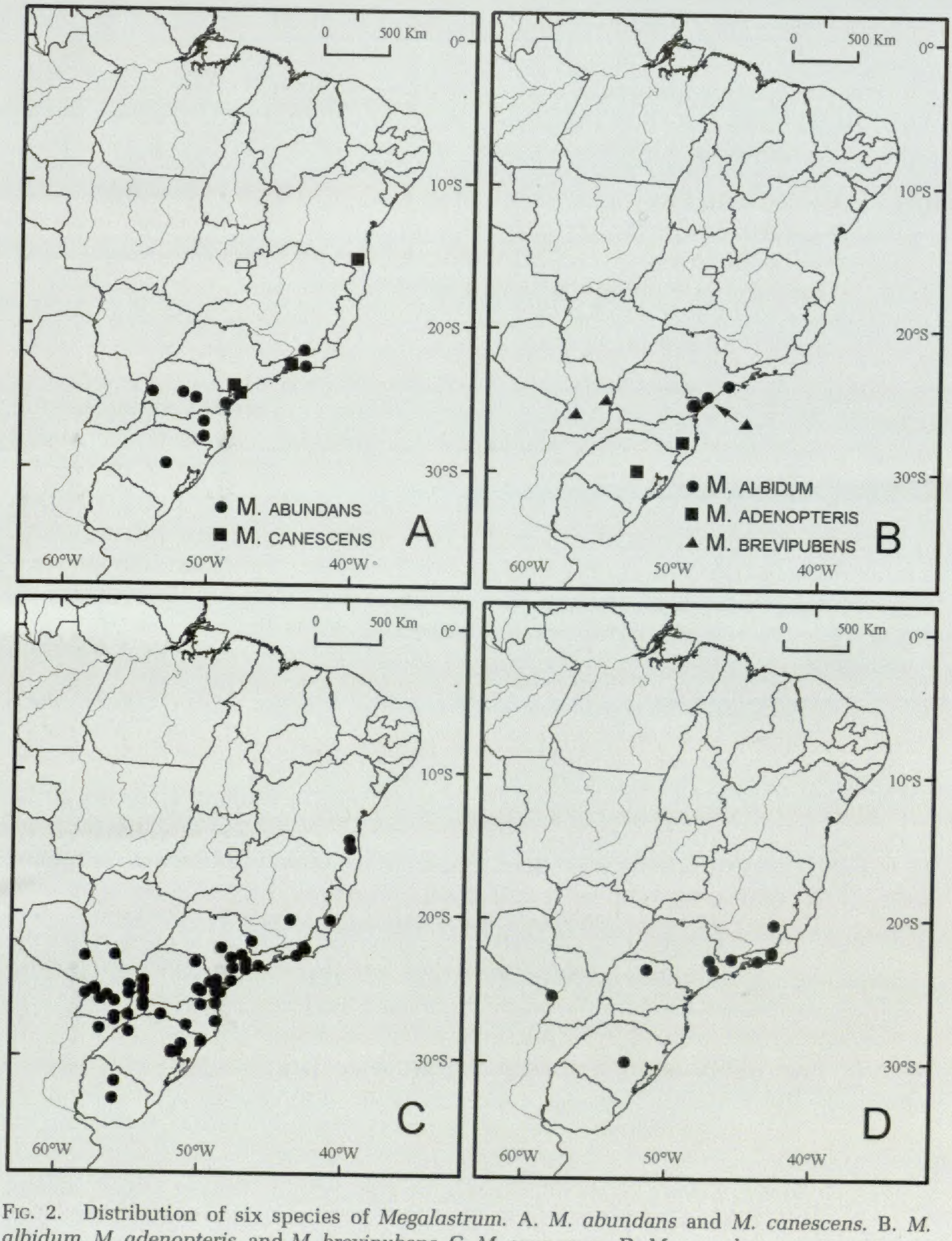

FIG. 2. Distribution of six species of *Megalastrum*. A. *M. abundans* and *M. canescens*. B. *M. albidum*, *M. adenopteris*, and *M. brevipubens*. C. *M. connexum*. D. *M. crenulans*.

Dryopteris martiana Rosenst., Hedwigia 46: 132. 1907. LECTOTYPE (here designated).—Brazil. **Paraná:** Villa Nova, 1906, *Annies 71* (Rosenstock Filices Austrobrasilienses no. 117) (S-1691; duplicates: B-n.v., BM, GH, MICH, NY, P, R-n.v., S, U, UC, US).

Leaves to 2.5 m long; *scales of petiole bases* 1–3 × 0.2–0.35 cm, linear, sparsely denticulate, brown; *laminae* 1–2 m long, 4-pinnate-pinnatifid at base, 3-pinnate-pinnatifid medially; *proximal pinnae* ca. 0.7 m long, strongly inequilateral, pinnules acroscopically reduced toward bases of pinnae; *pinna rachises* abaxially non-glandular, sparsely pubescent, hairs 0.5 mm, 4- or 5-celled, adaxially non-glandular, densely pubescent, hairs 0.3–0.4 mm long, 5- to 7-celled, scales 0.8–1.0 mm long, bullate, brown, ovate-lanceolate; *costules* on abaxial surfaces non-glandular, pubescent, hairs 0.1–0.5 mm, 1–4-celled, sparsely scaly, scales 0.8–1.0 mm long, bullate, brown, ovate-lanceolate; *laminar tissue between veins* abaxially non-glandular, glabrous to sparsely pubescent, hairs ca. 0.1 mm long, 2-celled, erect, adaxially glabrous (sparse hairs only on veins); *veins* visible on both surfaces, abaxially non-glandular, pubescent and minutely scaly, hairs 0.2–0.3 mm long, 1- or 2-celled, scales ca. 0.3 mm long, uniseriate, appressed, reddish, adaxially non-glandular, sparsely pubescent, hairs 0.3–0.5 mm long, 1–3-celled; *lamina margins* ciliate, hairs ca. 0.1 mm long, 1-celled, glandular hairs absent; *indusia* absent.

Distribution and ecology.—Endemic to coastal Brazil; 600–1200 m.

Additional Specimens Examined.—BRAZIL. **Minas Gerais:** Juiz de Fora, Poço Danta, [21°45′S, 43°21′W], Jul 1902, *Schwacke 14801* (P). **Paraná:** Pitanga, Borboleta, 24°45′S, 51°45′W, 600 m, 13 Dec 1973, *Hatschbach 33505* (C, UC); Piraquara, Mananciais da Serra, Morro do Canal, 25°30′58″S, 48°48′20″W, 1200 m, 20 Oct 2003, *Labiak et al. 3002* (NY, UC, UPCB). **Rio de Janeiro**: Serra da Estrela, [22°53′S, 43°13′W], *Luetzelburg s.n.* (S). **Santa Catarina:** Blumenau, 27°56′S, 49°03′W, 850–900 m, 3 Apr 2008, *Schwartsburd & Ceolin 1631* (NY, SP); Lages, [27°48′S, 50°18′W], Mar 1912, *Spannagel 324* (HBR, S, SP).

Megalastrum abundans is characterized by bullate scales on the pinna rachis and costules abaxially, lamina tissue glabrous adaxially between veins, veins pubescent adaxially, and non-indusiate sori (Fig. 8A–H). It and *M. crenulans* are the most finely divided species in the area, with laminae 4-pinnate (or more) at their bases (Fig. 5A). *Megalastrum abundans* differs from *M. crenulans* by lack of indusia.

2. ***Megalastrum adenopteris*** (C. Chr.) A. R. Sm. & R. C. Moran, Amer. Fern J. 77: 127. 1987. *Dryopteris adenopteris* C. Chr., Kongel. Danske Vidensk. Selsk. Skr., Naturvidensk. Math. Afd., ser. 8, 6: 85. 1920. *Ctenitis adenopteris* (C. Chr.) Ching, Sunyatsenia 5: 250. 1940. LECTOTYPE (here designated).—BRAZIL. **Rio Grande do Sul**: Silveira Martins, Val Veneta, ad terram silvae primaevae, in 1893, *Lindman s.n.* (Regnell A 1313) (BM-00907710; duplicates: BM, C-n.v., L-n.v., S-n.v., U-n.v., US; fragm. MO; photo MICH ex BM). **Figs. 2B**, **6F**, **10G–O**.

Dryopteris villosa (L.) Kuntze var. *tomentosa* Rosenst., Hedwigia 46: 130. 1916. LECTOTYPE (here designated).—BRAZIL. **Rio Grande do Sul**: Mun. Rio Pardo, Fazenda Soledade, [29°59′23″S, 52°22′41″W], 1906, *Jürgens s.n.* (Rosenstock Filices Austrobrasilienses no. 207) (MICH; duplicate: S-n.v.).

Dryopteris oreocharis Sehnem var. *canescens* Sehnem, Fl. Ilustr. Catarinense ASPI 1: 177. 1979. LECTOTYPE (here designated).—BRAZIL. **Santa Catarina**: Lauro Müller, Novo Horizonte, 400 m, 24 Oct 1958, *Reitz & Klein 7516* (PACA; duplicate: HBR).

Leaves to 4 m long; *scales of petiole bases* 1–2 × ca. 0.1 cm, linear, sparingly denticulate (nearly entire), light brown; *laminae* 1–2 m long, 4-pinnate at base, 3-pinnate-pinnatifid medially; *basal pinnae* ca. 1 m long, strongly inequilateral, pinnules acroscopically reduced toward bases of pinnae; *pinna rachises* abaxially glandular with sessile to short-stalked glandular hairs, adaxially pubescent and glandular, hairs 0.1–0.3 mm long, 1–3 celled; *costules* on abaxial surfaces sparsely scaly, scales 0.5–0.7 mm long, light brown, ovate-lanceolate, non-bullate, pubescent, hairs ca. 0.1–0.2 mm long, with many gland-tipped hairs (more so than on tissue between veins), glandular cell yellowish pubescent, hairs on adaxial surfaces 0.3–0.4 mm long, 3- or 4-celled; *laminar tissue between veins* densely and evenly puberulent on both surfaces; hairs on abaxial surfaces ca. 0.1 mm long, erect, 1-celled, some of the hairs gland-tipped, glandular cell yellowish, spherical, sometimes sessile or nearly so; hairs adaxially on tissue between veins, ca. 0.2 mm long, 1-celled, glandular hairs sparse; veins visible, pubescent on both surfaces, hairs abaxially ca. 0.3 mm long, 2-celled, hairs adaxially sparser, ca. 0.5 mm long, 3- or 4-celled; *lamina margins* ciliate, hairs 0.2–0.3 mm long, 1-celled, glandular hairs sparse to absent; *indusia* < 0.3 mm, fugacious and usually seemingly absent, glandular and pubescent, hairs sometimes appearing mixed among the sporangia.

Distribution and ecology.—Brazil, Argentina; 500–850 m.

ADDITIONAL SPECIMENS EXAMINED.—BRAZIL. **Rio Grande do Sul:** Val Veneta, 1893, *Lindman s.n.* (BM, MO, US); Rio Pardo, Fazenda Soledade, [29°59′22″S, 52°52′41″W], 1906, *Jürgens s.n.* [Rosenstock Filices Austrobrasilienses no. 207] (MICH).

Megalastrum adenopteris is characterized by dense even uniform, erect hairs on the abaxial surfaces of the laminae, glands on both surfaces of the laminae, pinna rachises, and costules, and minute fugacious indusia (Fig. 10G–O). Glands are usually most evident on the pinna rachises and costules abaxially, either sessile or with a one-celled stalk. Unlike most *Megalastrum* in the region, the rachis scales are widely spreading. The indusia are often apparently absent, or they appear as a cluster of several minute (ca. 0.05 mm long) scales. Whether this condition is homologous with true indusia is uncertain.

The most similar species is *Megalastum umbrinum*, which differs by laminae glabrous adaxially between the veins (or with a few hairs near the

margin), rachis scales appressed, and lack of minute fugacious indusia (Fig. 10A–F). *Megalastrum abundans* differs by numerous bullate scales on the pinna rachises and costules abaxially, sparser pubescence of the laminae abaxially, absence of glandular hairs, and lack of indusia (Fig. 8A–H). *Megalastrum crenulans* differs by persistence large indusia, the hairs between the veins abaxially all short (ca. 0.1 mm long) glandular, and hairs on the adaxial surfaces of the costules 0.5–0.7 mm long, 5- or 6-celled (Fig. 10P–AA).

3. *Megalastrum albidum* R. C. Moran, J. Prado & Labiak, ***sp. nov.*** TYPE.—BRAZIL. **Paraná**: Mun. Morretes, Serra da Graciosa, Caminho dos Jesuítas, 29°59′23″S, 52°22′41″W, 1000 m, 20 Oct 2003, *Labiak & Goldenberg 3011* (holotype: UPCB; isotypes: NY, SP). **Figs. 2B**, **7A**, **9E–H**.

A M. canescenti *laminis ad basim 3-pinnato-pinnatisectis, ad medium 2-pinnato-pinnatisectis, utrinque pubescentibus atque rachidibus pinnarum abaxialiter squamis linearibus et pilis 0.3–0.6 mm longis albidis vestita differt.*

Leaves to 2 m long; *scales of the petiole bases* ca. 1.5–2 × 0.04–0.1 cm, linear, sparsely denticulate, yellowish to golden brown, flat (not twisted), en masse not forming a woolly tuft; *laminae* 1.5 m long, to 3-pinnate-pinnatisect at base, 2-pinnate-pinnatifid medially; *basal pinnae* to 45 cm long, stalks to 2 cm long, strongly inequilateral, pinnules acroscopically slightly reduced toward the bases of pinnae; *pinna rachises* abaxially non-glandular, sparsely pubescent, very sparsely scaly, hairs 0.3–0.6 mm long, 3–5-celled, scales 1–2 mm long, linear, brown, denticulate, flat (not bullate), slightly spreading, adaxially non-glandular, densely pubescent, hairs ca. 0.3–0.6 mm long, 3–5-celled, patent (not strigose); *costules* abaxially non-glandular, pubescent, hairs of relatively uniform length, ca. 0.8–1.0 mm long, 5–8-celled, acicular, whitish, scaly, scales small (ca. 0.5–1 mm long), subentire to denticulate, linear, subappressed, adaxially non-glandular, pubescent, hairs ascending to antrorsely strigose, ca. 0.5 mm long, 3–5-celled; *laminar tissue between veins* abaxially non-glandular, densely pubescent, sparsely scaly, hairs ca. 1–1.5 mm long, 6–9-celled, erect, scales ca. 0.1 mm long, uniseriate, linear, appressed, reddish, inconspicuous, adaxially pubescent, hairs 0.4–0.7 mm long, 4–7-celled, spreading to erect, very sparse scaly, scales ca. 0.2–0.4 mm long, uniseriate, appressed, light reddish, both surfaces dull; *veins* visible on both surfaces, non-glandular, pubescent and scaly, hairs ca. 1–1.5 mm long, 6–9-celled, adaxially densely pubescent, hairs 0.4–0.7 mm long, 4–7-celled; *lamina margins* ciliate, non-glandular, hairs ca. 0.2–0.3 mm long, 2–3-celled; *indusia* absent.

Distribution and ecology.—SE Brazil (Paraná and São Paulo); 0–1000 m.

Additional Specimens Examined.—BRAZIL. **Paraná:** Três Barras, 27 Jan 1916, *Dusén 17563* (S). **São Paulo:** Iguape, Serra de Itatins, [24°42′28″S, 47°47′18″W], 600 m, Mar 1924, *Brade s.n.* (US); Santos, [23°57′S, 46°20′W], 15 Nov 1874, *Mosén 3091* (S).

Megalastrum albidum has linear scales on the pinna rachises abaxially, these mixed with whitish hairs 0.4–0.6 mm long (Fig. 9E–H). Also characteristic are the laminae pubescent on both surfaces between the veins. It resembles *M. canescens* (Fig. 12A–F), a species that differs by glandular, stalked hairs on the abaxial lamina surfaces, hairs of mixed length on the costules, and generally shorter hairs on the laminar tissue between the veins (shorter than those along the veins). Furthermore, the laminae of *M. canescens* are 2-pinnate-pinnatisect at base, 2-pinnate-pinnatifid medially (Fig. 5B), whereas the laminae of *M. albidum* are 3-pinnate-pinnatisect at base, 2-pinnate-pinnatifid medially (Fig. 7A).

Megalastrum albidum is named for the dull whitish hairs on both surfaces of the laminae.

4. *Megalastrum brevipubens* R. C. Moran, J. Prado & Labiak, ***sp. nov.*** TYPE.—PARAGUAY. **Amambay**: Sierra de Amambay [ca. 23°00′S, 58°00′W, 300 m], Sep 1907, *Hassler 10802* (holotype: NY; isotypes: BM, K, MICH). **Figs. 2B, 7F, 11F–K.**

A M. connexo *lamina abaxialiter pilis acicularibus 0.1–0.2 mm longis erectis inter venas vestita differt.*

Leaves ca. 1.0 m long; *scales of petiole bases* ca. 2 × 0.15 cm, linear to lanceolate, sparsely denticulate, light brown to yellowish or golden, twisted or crispate, en masse forming a dense wool; *laminae* ca. 75 cm long, to 3-pinnate-pinnatifid at base, 2-pinnate medially; *basal pinnae* ca. 30–50 cm long, stalks to 2 cm long, strongly inequilateral, pinnules acroscopically slightly reduced toward the base of pinna; *pinna rachises* abaxially non-glandular, glabrous or nearly so, with a few (usually at pinna base) scales, these 2.5 mm long, linear, denticulate, adaxially densely pubescent, non-glandular, hairs 0.1–0.3 mm long, 1–4-celled; *costules abaxially* non-glandular, pubescent to lacking hairs, sparsely scaly, hairs 0.2–0.4 mm long, 2–4-celled, scales 1–2 mm long, filiform to narrowly lanceolate, non-bullate, adaxially pubescent, hairs 0.1–0.6 mm long, 3–5-celled; *laminar tissue between veins* abaxially non-glandular, puberulent, hairs ca. 1 mm, 1-celled, erect to substrigose, sometimes with sparse uniseriate scales, these ca. 0.2 mm long, appressed, brown, glabrous adaxially; *veins* adaxially pubescent, visible, abaxially pubescent, hairs 0.2–0.3 mm long, 1–3-celled, with sparse uniseriate, filiform scales, these ca. 0.2 mm long, appressed, brown; *lamina margins* non-glandular, sparsely ciliate, hairs ca. 0.1–0.2 mm long, 1(2)-celled; *indusia* absent.

Distribution and ecology.—Brazil, E Paraguay; [200–300 m].

Additional Specimens Examined.—BRAZIL. **São Paulo:** Sete Barras, Fazenda Intervales, Base de Saibadela, 20 Jul 1994, *Salino 1987* (AAU). **Unknown:** *Burchell 3160* (K); 30 Mar 1883, *Urban s.n.* (MICH).

PARAGUAY. **Guaira:** Tororo, Arroyo Polilla, 25°55′S, 56°56′W, 14 Dec 1988, *Soria 2848* (MO).

Megalastrum brevipubens is distinctive by the short (ca. 0.1 mm long), erect, acicular hairs between the veins on the abaxial surfaces of the laminae (Fig. 11F–K). It resembles *M. connexum*, a species that is glabrous between the veins abaxially (Fig. 11L–P).

5. *Megalastrum canescens* (Kunze ex Mett.) A. R. Sm. & R. C. Moran, Amer. Fern J. 77: 127. 1987. *Phegopteris canescens* Kunze ex Mett., Abh. Senckenberg. Naturf. Ges. 2: 314. 1858. *Polypodium villosum* L. var. *canescens* Baker, Flora Brasil 1(2): 483. 1870. *Ctenitis canescens* (Kunze ex Mett.) C. V. Morton, Contr. U.S. Natl. Herb. 38: 54. 1967. LECTOTYPE (here designated).—BRAZIL. **Bahia**: Castelnovo, 1836, *Blanchet 2454* (NY; B-n.v.; isotypes: GH, K, P). **Figs. 2A**, **5B**, **12A–F**.

Phegopteris cana Mett., Ann. Mus. Bot. Lugduno-Batavi 1: 223. 1864. TYPE.—BRAZIL. s.d., *collector unknown* (B-n.v.). [Placed here in synonymy following Christensen, 1920].

Phegopteris mollivillosa Fée, Mém. Soc. Sci. Nat. Strasbourg [Mém. Foug. 10] 32. 1865. LECTOTYPE (here designated).—BRAZIL. State unknown: s.d., *Martius 320* (P-00600562, 00600563; duplicate: BM).

Leaves to 2 m long; *scales of petiole bases* ca. 1 × ca. 0.1 cm, lanceolate, sparsely denticulate, brown to dark brown, sometimes with blackish denticulate margins (this color often absent on scales on the distal portion of petiole); *laminae* 1–2 m long, 2-pinnate-pinnatisect at base, 2-pinnate medially; *basal pinnae* ca. 0.4 m long, stalks to 3.5 cm long, strongly inequilateral, pinnules acroscopically reduced toward pinna bases; *pinna rachises* abaxially glandular, pubescent and sparsely scaly (scales sometimes apparently absent), glands ca. 0.1 mm long, hairs ca. 1 mm long, 5–7-celled, scales ca. 1.5 mm, non-bullate, ovate-lanceolate, subentire, apices long-acuminate; adaxially apparently non-glandular, densely pubescent, hairs ca. 1.2 mm long, 5- or 8-celled; *costules* glandular, glands 0.1 mm long, pubescent abaxially with two sizes of hairs, longer ones ca. 1 mm long, 3–5-celled, shorter ones ca. 0.2 mm long, 1- or 2-celled, sparsely scaly, scales to 1 mm long, uniseriate, appressed, brown; *laminar tissue between veins* abaxially glandular and pubescent, glands ca. 0.1 mm long, 1- or 2-celled, yellowish, abundant to nearly absent, hairs 0.3–0.5 mm long, 2- or 3-celled, adaxial surfaces sparsely pubescent, hairs 0.2–0.4 mm long, 1- or 2-celled, appressed; *veins* visible and pubescent on both surfaces, hairs on abaxial surfaces 0.4–0.8 mm long, 1–3-celled, adaxial surfaces with hairs 0.5–0.8 mm long, 3–6-celled, glandular hairs absent; *lamina margins* ciliate, hairs ca. 0.4–0.5 mm long, 1- or 2-celled, glandular hairs apparently absent; *indusia* absent.

Distribution and ecology.—SE Brazil; 600–1200 m.

Additional Specimens Examined.—BRAZIL. **Bahia:** Camacan, Ramal para a Torre da Embratel na Serra Boa, N de São João da Panelinha, [15°25′8″S, 39°39′45″W], 6 Apr 1979, *Mori & dos Santos 11703* (K, NY, US); Camacan,

RPPN Serra Bonita, 9.7 km de Camacan na estrada para Jacarecí, daí 6 km SW na estrada para a RPPN e torre da Embratel, 15°23′30″S, 39°33′55″W, 835 m, 3 Mar 2006, *Matos et al. 1094* (CEPEC, UPCB). **Rio de Janeiro:** Bico do Papagaio, 600 m, 4 Jun 1929, *Brade s.n.* (US); Parati, Parque Nacional da Serra da Bocaina, 23°11′22″S, 44°50′15″W, 1200 m, 7 Jan 2008, *Labiak et al. 4372* (NY, SP, UPCB). **São Paulo**: Iguape, Morro das Pedras, [24°42′28″S, 47°47′18″W], Oct 1920, *Brade 7713* (GH, HB, NY, S, UC, US); Rio Ipiranga confluência com Rio Juquiá, [24°20′29″S, 47°47′44″W], Oct 1925, *Brade 21315* (HB, GH). **Unknown:** *Riedell 51* (GH).

Megalastrum canescens is characterized by hairs ca. 1 mm long and 5–8-celled on the abaxial surfaces of the laminae, intermixed with shorter ones, up to 0.2 mm long, non-bullate scales, and lack of indusia (Fig. 12A–F). The petiole base scales of this species tend to have blackish margins. Superficially, this species resembles *M. albidum*, which see for comparison.

6. ***Megalastrum connexum*** (Kaulf.) A. R. Sm. & R. C. Moran, Amer. Fern J. 77: 128. 1987. *Polypodium connexum* Kaulf., Enum. Filic. 120. 1824. *Phegopteris connexa* (Kaulf.) Fée, Gen. Filic. 243. 1852. *Dryopteris connexa* (Kaulf.) C. Chr., Index Filic. 258. 1905. *Nephrodium connexum* (Kaulf.) Hicken, Revista Mus. La Plata ser. 2, 15: 231. 1908. *Ctenitis connexa* (Kaulf.) Copel., Ann. Crypt. Phytopath. [Gen. Fil.] 5:124. 1947. NEOTYPE (designated here): BRAZIL. **Santa Catharina**: Ilha de Santa Catarina (Florianópolis), Sertão da Lagoa, [27°35′49″S, 48°48′56″W], 26 Jun 1948, *Rohr 1071* (US; isoneotypes: HB, HBR, NY). **Figs. 2C**, **5D**, **6D**, **11L–P**.

Phegopteris brevinervis Fée, Mém. Foug. 10: 32. 1965. TYPE.—BRAZIL. State Unknown: s.d., *Claussen s.n.* (holotype: P).

Phegopteris eriopoda Fée, Crypt. Vasc. Brésil 1: 102, t. 31, fig. 1. 1869. LECTOTYPE (here designated).—BRAZIL. **Rio de Janeiro**: Rio de Janeiro, Gavia sur les bords du Chemin, [22°56′S, 43°17′W], 12 Mar 1868, *Glaziou 2397* (P-00610813; duplicates: C, S, photos MICH, MO ex C).

Phegopteris adnata Fée, Crypt. Vasc. Brésil 1: 103. 1869. LECTOTYPE (here designated).—BRAZIL. **Rio de Janeiro**: Rio de Janeiro, Alto Macaé, [22°56′S, 43°17′W], 21 May 1868, *A. Glaziou 2398* (P-00610860; duplicates: C; photo MICH ex C).

Phegopteris propinqua Fée, Crypt. Vasc. Brésil 1: 103, t. 32, fig. 3. 1869. TYPE.—BRAZIL. **Rio de Janiero**: Rio de Janeiro, Fazenda do Ariro [22°56′S, 43°17′W], 28 Jun 1868, *Glaziou 2399* (Holotype: P).

Polypodium willsii Baker, Ann. Bot. 5: 458. 1891. *Dryopteris willsii* (Baker) C. Chr., Index Filic. 301. 1905. TYPE.—BRAZIL. **Rio de Janeiro**: Rio de Janeiro, [22°56′S, 43°17′W], Jun 1881, *Wills s.n.* (holotype: K).

Phegopteris lateadnata H. Christ, Ann. Cons. Jard. Bot. Genéve 3: 36 1899. *Dryopteris lateadnata* (H. Christ) C. Chr., Index Filic. 274. 1905 [as "*D. latealata*," a typographical error]. *Dryopteris connexa* var. *lateadnata* (H. Christ) C. Chr., Kongel. Danske Vidensk. Selsk. Skr., Naturvidensk. Math.

Afd., ser. 8, 6: 80. 1920. LECTOTYPE (here designated).—PARAGUAY. Caaguazu: 1 Apr 1885 s.d., *Balansa 313a* (holotype: P-00610679; duplicates: B-n.v., K, L-n.v., P).

Leaves to 1.5–2.5 m long; *scales of the petiole bases* ca. 2 × 0.15 cm, linear to lanceolate, sparsely denticulate, light brown to yellowish or golden, twisted or crispate, en masse forming a dense wool; *laminae* 1–2 m long, to 3-pinnate at base, 2-pinnate medially; *basal pinnae* ca. 30–50 cm long, stalks to 2 cm long, strongly inequilateral, pinnules acroscopically slightly reduced toward pinna bases; *pinna rachises* abaxially non-glandular, glabrous or nearly so, with a few (usually at pinna base) scales, these 2.5 mm long, linear, denticulate, adaxially densely pubescent, non-glandular, hairs 0.1–0.3 mm long, 1–4-celled; *costules* abaxially non-glandular, pubescent or lacking hairs, sparsely scaly, hairs 0.2–0.3 mm long, 2–4-celled, scales 1–2 mm long, filiform to narrowly lanceolate, non-bullate, adaxially pubescent, hairs 0.1–0.6 mm long, 3–5-celled; *laminar tissue between veins* abaxially non-glandular, glabrous, sometimes with sparse uniseriate scales, these ca. 0.2 mm long, appressed, brown, adaxially glabrous; *veins* adaxially glabrous or with scattered hairs, visible, abaxially glabrous or pubescent, hairs ca. 0.2 mm long, 1–3-celled, with sparse uniseriate, filiform scales, these ca. 0.2 mm long, appressed, brown; *lamina margins* non-glandular, sparsely ciliate or apparently eciliate, hairs ca. 0.1 mm long, 1(2)-celled; *indusia* absent.

Distribution and ecology.—Brazil, Argentina, Paraguay, and Uruguay; 0–1000 m.

Additional Specimens Examined.—BRAZIL. **Bahia:** Camacan, RPPN Serra Bonita, 9.7 km de Camacan, Fazenda Serra Bonita, 9.7 km W de Camacan na estrada para Jacarecí, dai 6 km SW na estrada para a RPPN e torre da embratel, 15°23′30″S, 39°33′55″W, 835 m, 13 Feb 2005, *Matos et al. 439* (UPCB); Ilhéus, [14°47′S, 39°39′W], *Moricand 2469* (K). **Espírito Santo:** Concórdia, Concórdia a Cachoeira, 1889, *Bello 533* (R). **Minas Gerais:** Serra de Caldas, [17°44′S, 48°37′W], 10 Sep 1873, *Mosén 2184* (P, S, U). Serra de Ouro Preto, 07 Jan 1894, *Schwacke 10230* (P). **Paraná**: Vila Nova, [24°42′50″S, 53°53′34″W], Mar 1905, *Annies s.n.* [Rosenstock Filicies Austrobraslienses 100](MICH, S); Campo Mourão, Mata ao lado da Usina Mourão, 24°07′06″S, 52°19′13″W, 24 Dec 2007, *Labiak et al. 4260, 4262* (NY, SP, UPCB). **Rio de Janeiro:** Gavla sur les bords du chemin, 12 Mar 1868, *Glaziou 2397* (C, P, S); Alto Macaé, 21 May 1868, *Glaziou 2398* (C, P). **Rio Grande do Sul**: São Leopoldo, [29°45′36″S, 51°51′49″W], 23 Apr 1905, *Eugênio 39* (NY); Parecí Novo. Montenegro, 100 m, 13 Oct 1945, *Sehnem 1343* (US). **Santa Catarina:** Florianópolis, Sertão da Lagoa, [27°35′49″S, 48°48′56″W], 26 Jun 1948, *Rohr 1071* (HB, HBR, NY, US); Chapecó, Seminário Diocesiano, W of Chapecó, 27°06′S, 52°37′W, 16 Dec 1964, *Smith & Klein 14041* (US). **São Paulo**: São Paulo, Serra da Cantareira, [23°22′S, 46°46′W], 11 Jan 1914, *Brade & de Toledo 6634* (GH, HB, NY, UC); Iguape, Morro das Pedras, [24°42′28″S, 47°47′18″W], Aug 1916, *Brade 7714* (GH, HB, K, NY, S, UC). **Unknown:** *Burchell 1915* (GH, K, P); *Glaziou 1780* (C, P); *Glaziou 7948* (C, P).

PARAGUAY. **Alto Paraná:** Reserva Biológica de Itabó, 35 km W Río Paraná, 25°5′S, 54°54′W, 10 Oct 1990, *Schinini & Marmori 27051* (GH); Estancia Río Bonito, Forest I, 25°38′50″S, 54°54′45″W, 240 m, 29 Aug 1994, *Zardini & Vera 40653* (MO). **Amambay:** In altapanitie et declivibus, Sierra de Amambay, [22°56′S, 55°55′W], 1907–1908, *Hassler 10278* (BM, MICH, P); In altapanitie et declivibus, Sierra de Amambay, [22°56′S, 55°55′W], 1907–1908, *Hassler 10802* (B, K, MICH, NY). **Caazapa:** Tavai Pacuri, Comunidad Mbya, 26°10′S, 55°55′W, 22 Dec 1988, *Basualdo 2127* (MO); Parque Nacional de Caaguazú, Bosque de hasta 20 m de altura, Linea del Parque hacia ao Itá y ao Jaku'y Ruta Prov. 101, camino al aeropuerto, sobre el desvio a las Cataratas del Iguazú, [28°6′60″S, 56°56′60″W], 22 Jul 1986, *Molas 823* (MO). **Central:** in regione Lacus Ypacaray, [25°23′S, 57°16′W], *Hassler 12944* (P). **Paraguarí:** prope Sapucay, [26°3′S, 56°56′W], Aug 1913, *Hassler 12203* (C, K, MO, P, S, US); Parque Nacional Ybycu'í, Sendero dentro del bosque ribereno, [26°3′S, 56°56′W], 31 May 1987, *Zardini & Pérez 2881* (MO). **San Bernardino:** [25°16′S, 57°19′W], 2 Sep 1915, *Osten 8435* (S). **Unknown:** Apr 1881, *Balansa 2910* (BM, C, P, S); 1885–1895, *Hassler 1841* (BM, K, NY, P); 1885–1895, *Hassler 660b* (BM, K, NY, P, S).

URUGUAY. **Rivera:** Tranqueras, [31°0′S, 46°0′W], 7 May 1945, *Legrand 3994* (US). **Tacuarembó:** Sierra del Tacuarembó, gruta de los helechos, [33°00′S, 56°00′W], 300 m, 24–28 Aug 1907, *Herter 3534a* (P).

Megalastrum connexum is characterized by glabrous pinna rachises abaxially and filiform scales on the costules and veins abaxially (Fig. 11L–P). Both surfaces of the lamina between the veins are glabrous. The costular indument is variable. In all specimens there are hairs and scales on the costules abaxially, but some specimens have more scales than hairs, and vice-versa. Judging from the number of specimens, this is one of the most common species in Brazil.

Megalastrum brevipubens has similar laminar cutting but differs by minute (ca. 0.1 mm long), erect, acicular hairs on the laminar tissue between the veins (Fig. 11F–K).

Two specimens from Paraguay (*Hassler 12203, 12942a*) are unusual in lamina cutting. The pinnae are extremely large, with ultimate segments widely spaced and slightly acute and falcate; however, we find no differences in the indument with typical plants, and no other differences correlate with lamina division. Therefore, we consider the specimens part of the variation within *M. connexum*.

Both Christensen (1920) and we were unable to find Kaulfuss's type specimen of *Polypodium connexum* (Brazil, Sta. Catarina, s.d., *Chamisso s.n.*), which should be housed at B or LE. For this reason, we are neotypifying this long-used name. The neotype chosen is from the island of Santa Catarina where the Chamisso specimen was collected.

7. ***Megalastrum crenulans*** (Fée) A. R. Sm. & R. C. Moran, Amer. Fern J. 77: 127. 1987. *Aspidium crenulans* Fée, Crypt. Vasc. Brésil 1: 139, t. 47, fig. 1. 1869. *Dryopteris crenulans* (Fée) C. Chr., Kongel. Danske Vidensk. Selsk.

Skr., Naturvidensk. Math. Afd., ser. 8, 6: 90. 1920. *Ctenitis crenulata* (Fée) Ching, Sunyatsenia 5: 250. 1940. LECTOTYPE (designated by Christensen, 1920): BRAZIL. **Rio de Janiero**: Rio de Janeiro, [22°56′S, 43°17′W], *Glaziou 1781* (C; duplicates K, P, RB-n.v.; photos MICH, MO ex C). **Figs. 2D**, **5D**, **10P–AA**.

Dryopteris villosa var. *glandulosa* Rosenst., Hedwigia 46: 129. 1907. *Dryopteris crenulans* forma *glandulosa* (Rosenst.) C. Chr., Kongel. Danske Vidensk. Selsk. Skr., Naturvidensk. Math. Afd., ser. 8, 6: 91. 1920. LECTOTYPE (here designated).—BRAZIL. **Rio Grande do Sul**: Mun. Rio Pardo, banks of Rio Cyriaco, 1906, *Jürgens s.n.* [Rosenstock Filices Austrobrasilienses no. 206] (BM-000907729; duplicates: GH, HB, K, MICH, MO, NY, P, S-n.v., UC).

Leaves to 2.5 m long; *scales of the petiole bases* ca. 2 × ca. 0.1 cm, linear, sparsely denticulate, light brown, twisted, en masse forming a dense woolly tuft; *laminae* 1 m long, to 4-pinnate at base, 2-pinnate-pinnatifid medially; *basal pinnae* ca. 30–50 cm long, stalks to 2.5 cm long, strongly inequilateral, pinnules acroscopically slightly reduced toward the pinna bases; *pinna rachises* abaxially glandular, pubescent, hairs 0.8–1.2 mm long, 4- or 5-celled, densely glandular, glands ca. 0.1 mm long, 2-celled, scaly, scales ca. 1 mm long, lanceolate, subentire, flat (non-bullate), adaxially densely pubescent, hairs ca. 1 mm long, 3–6-celled, non-glandular; *costules* abaxially glandular, pubescent, hairs 0.3–0.4 mm long, 1–3-celled, glands ca. 0.1 mm, 2-celled, scaly, scales ca. 1 mm long, bullate, subentire, adaxially pubescent, hairs 0.4–0.8 mm long, 1–3-celled, sparsely glandular, glands like those on the abaxial surfaces; *laminar tissue between veins* abaxially densely glandular and pubescent, hairs ca. 0.1 mm long, 1-celled, adaxially glandular but slightly less so than abaxially, hairs absent; *veins* visible on both surfaces, very sparsely glandular abaxially, pubescent abaxially, hairs 0.2–0.3 mm long, 1- or 2-celled, adaxially sparsely pubescent, hairs ca. 0.4–0.5 mm long, 1–3-celled; *lamina margins* densely ciliate, hairs ca. 0.3–0.4 mm long, 1- or 2-celled; *indusia* present, circular, dark brown, glandular, pubescent, or both, hairs 0.3–0.4 mm long, 1- or 2-celled.

Distribution and ecology.—Venezuela, Brazil, and Paraguay; 725–1760 m.

Additional Specimens Examined.—BRAZIL. **Minas Gerais:** Viçosa, Fazenda de Aguada, [20°47′45″S, 50°50′W], 725 m, 17 Sep 1930, *Mexia 5060* (NY, S, UC, US). **Paraná:** Apucarana, Pirapó, [23°33′S, 51°26′W], Jun 1951, *Tessmann 593* (HB). **Rio de Janeiro:** Teresópolis, Quebra frascos, [22°24′44″S, 42°42′56″W], 17 Oct 1929, *Brade 9695* (GH, NY, UC); Fazenda da Santa Anna, [22°54′S, 43°43′W], *Glaziou 2351* (C, K, MO, P [syntypes]). **Rio Grande do Sul:** 1865, *Page s.n.* (US). **São Paulo:** São Paulo, Água Funda, nativa no Jardim Botânico, [23°32′52″S, 46°46′9″W], May 1973, *Handro 2224* (GH, HB, US); Campos do Jordão, [22°46′S, 45°31′W], Jul 1945, *Leite 3567* (UC).

PARAGUAY. **Central:** Cordillera Central, [25°22′60″S, 57°57′60″W], Dec 1900, *Hassler 6898* (GH, MICH, NY, P).

Megalastrum crenulans is nearly unique in the region by having a large persistent indusium (Fig. 10P–AA). It is further distinctive by glands on both surfaces of the laminae and bullate scales on the abaxial surfaces of the costules. The indusia are variable in the presence of hairs and glands, but no other character apparently correlates with this. The only other species with a well-defined indusium is *M. indusiatum*, but the indusium in that species is much smaller, only about the size of a single sporangial capsule, and are easily overlooked.

Fée (1869) applied the name *Aspidium consobrinum* Fée (=*Ctenitis*), type from Guadeloupe, to the Brazilian specimens *Glaziou 2350*, *979*, and *Gaulthier s.n.* In our opinion, these are typical *M. crenulans*.

8. *Megalastrum eugenii* (Brade) A. R. Sm. & R. C. Moran, Amer. Fern J. 77: 127. 1987. *Dryopteris eugenii* Brade, Rodriguésia 4(13): 298, t. 2. 1940. *Ctenitis eugenii* (Brade) Brade, Bradea 1(22): 209. 1972. LECTOTYPE (here designated).—BRAZIL. **Ceará**: Serra de Baturité, Sítio Santa Clara, [4°19′44″S, 38°53′06″W], 9 Dec 1937, *Eugênio s.n.* (US; duplicates: HBR, RB-n.v.). **Figs. 3A**, **7C**, **11Q–V**.

Leaves to 1.0–1.5 m long; *scales of the petiole bases* ca. 1.5 × 0.05–0.07 cm long, linear, sparsely denticulate, brown, slightly twisted, en masse forming a woolly tuft; *laminae* 1 m long, to 2-pinnate-pinnatifid at base (rarely 1-pinnate-pinnatisect), 1-pinnate-pinnatisect medially; *basal pinnae* 20–30 cm long, stalks to 1 cm long, inequilateral, pinnules acroscopically not or only slightly reduced toward pinna bases; *pinna rachises* abaxially non-glandular, glabrous to puberulent, sparsely scaly, hairs 0.1–0.2 mm long, 1–3-celled, scales 1–2 mm long, narrowly lanceolate, brown, denticulate, flat (non-bullate), adaxially non-glandular, pubescent, hairs 0.3–0.4 mm long, 2- or 3-celled, strigose; *costules* abaxially non-glandular, glabrous to puberulent, hairs 0.1–0.2 mm long, 1–3-celled, scaly, scales ca. 1 mm long, linear to narrowly lanceolate, non-bullate, sparsely denticulate, adaxially puberulous throughout, hairs 0.4–0.5 mm long, 1–3-celled; *laminar tissue between veins* abaxially non-glandular, glabrous to subglabrous, hairs (when present) ca. 0.2 mm long, 1- or 2-celled, uniseriate scales often present, these appressed, reddish, inconspicuous, adaxially non-glandular, glabrous to sparsely pubescent (often near margins), hairs 0.3–0.4 mm long, 2- or 3-celled; *veins* visible or obscure on both surfaces, non-glandular, glabrous to sparsely pubescent; *lamina margins* sparsely to densely ciliate, hairs 0.2–0.4 mm long, 2–3-celled; *indusia* absent.

Distribution and ecology.—Endemic to coastal NE Brazil (Alagoas, Bahia, Ceará, and Pernambuco); 600–700 m.

Additional Specimens Examined.—BRAZIL. **Alagoas:** Ibateguara, Engenho Coimbra, 9°00′S, 35°51′W, 380–400 m, 19 Dec 2000, *Pietrobom 4698* (HB, SP). **Bahia:** Arataca, Serra do Peito de Moça, estrada que liga Arataca a Una, ramal ca. 22,4 km de Arataca com entrada no Assentamento Santo Antônio,

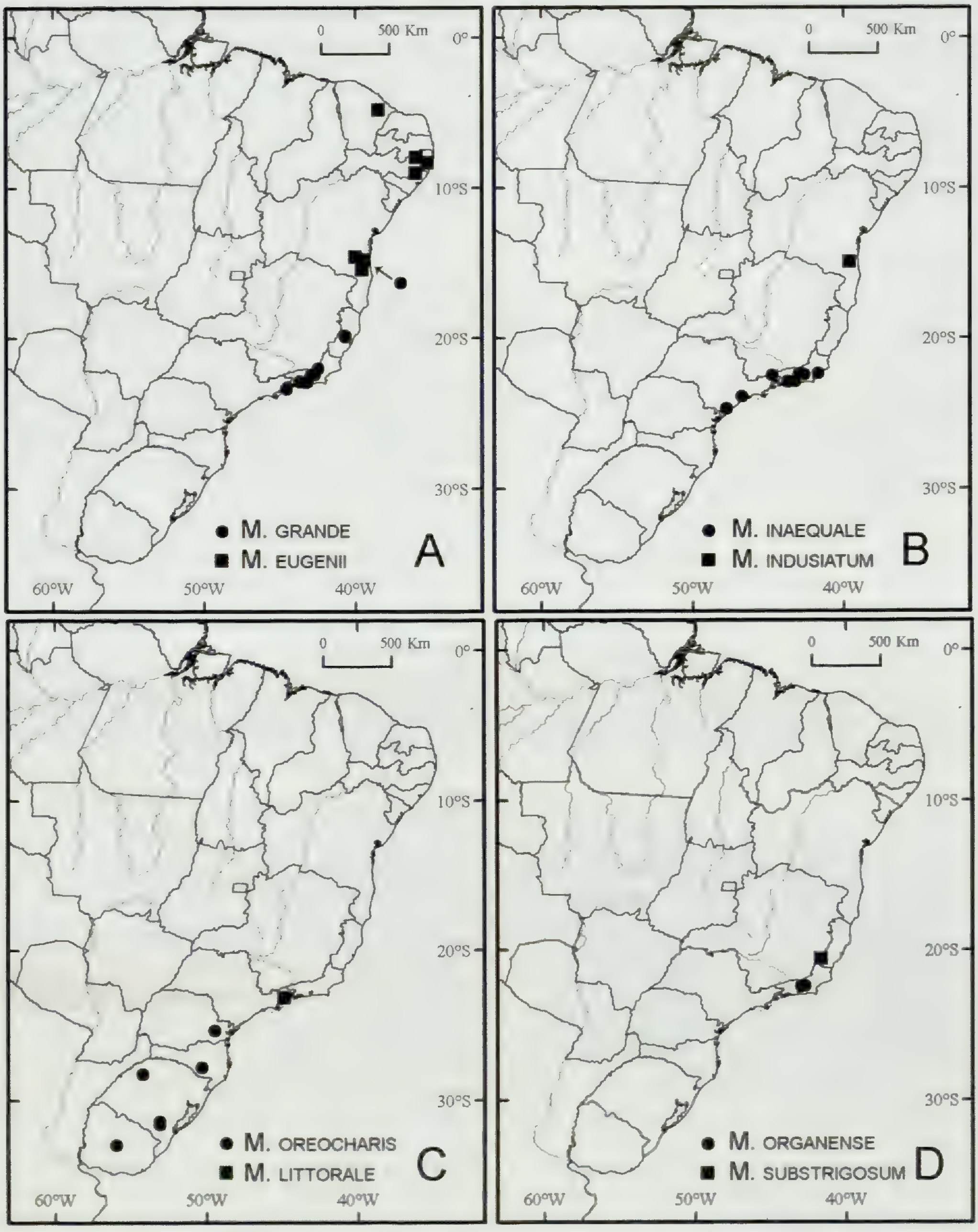

FIG. 3. Distribution of eight species of *Megalastrum*. A. *M. grande* and *M. eugenii*. B. *M. inaequale* and *M. indusiatum*. C. *M. oreocharis* and *M. littorale*. D. *M. organense* and *M. substrigosum*.

15°10′25″S, 39°20′30″W, 650 m, 6 Aug 2006, *Labiak et al. 3673, 3678* (CEPEC, NY, UPCB); prope São Pedro de Alcantara ad Vila dos Ilheos, [14°47′20″S, 39°39′56″W], 1839, *Luschnath Mart. Herb. Fl. Bras. 327* (BM, K, NY, P). **Pernambuco:** Timbaúba, Complexo do Mascarenhas, Mata do Estado, 7°37′S,

35°23′W, 304–394 m, 28 Apr 2001, *Pietrobom et al. 5225* (HB, SP); São Vicente Ferrer, Complexo do Mascarenhas, Mata do Estado, 7°35′0″S, 35°35′0″W, 600–650 m, 17 Aug 1998, *Pietrobom 4394* (NY).

Megalastrum eugenii resembles *M. grande* but differs by the presence of hairs on the costae adaxially (cf. Fig. 11Q–V and Fig. 9J–M). This is the only species that occurs in the northern part of northeastern Brazil.

9. *Megalastrum grande* (C. Presl) A. R. Sm. & R. C. Moran, Amer. Fern J. 77: 127. 1987. *Polypodium grande* C. Presl, Deliciae Pragenses 1: 171. 1822. *Dryopteris grandis* (C. Presl) C. Chr., Index Filic. 268. 1905. *Ctenitis grande* (C. Presl) Copel., Ann. Crypt. Phytopath. [Gen. Fil.] 5: 124. 1947. TYPE.—BRAZIL. **Rio de Janeiro**: collector unknown, Christensen (1920) suspected *J. E. Pohl s.n.* (holotype: PR-n.v.; isotype: W?-n.v.). **Figs. 3A, 5E, 9J–M.**

Polypodium auriculatum Raddi, Syn. Fil. Bras. 10 (no. 74). 1819 [seors. Praeimpr. ex Opusc. Sci. Bol. 3(5): 288. 1819], *nom. illeg.*, non L. 1753. *Polypodium formosum* Raddi, Pl. Bras. Nov. Gen. 1: 25, tab. 38. 1825, *nom. nov.* for *P. auriculatum* Raddi, non L. 1753. TYPE.—BRAZIL. **Rio de Janeiro**: Mt. Corcovado, [22°56′S, 43°17′W], s.d., *Raddi s.n.* [specimen no. 1 with "*Polypodium formosum nob.*" written by Raddi on the cover], (Lectotype: designated by Pichi Sermolli & Bizzarri, 2005): PI-n.v.; duplicates: FI-n.v., P, UC).

Polypodium macropterum Kaulf., Enum. Fil. 111. 1824. *Phegopteris macroptera* (Kaulf.) Fée, Gen. Filic. 243. 1852. *Polypodium splendidum* Kaulf., Enum. Fil. 112. 1824. *Phegopteris splendida* (Kaulf.) Fée, Gen. Filic. 243. 1852. *Polypodium macropterum* Kaulf. var. *splendida* Baker, Fl. Bras. 1(2): 502. 1870. LECTOTYPE (here designated).—BRAZIL. **Rio de Janeiro**, [22°56′S, 43°17′W], s.d., *Mertens s.n.* (C; duplicates: B-n.v.; photos MICH, MO, NY ex C).

Polypodium repandum Vell., Flora Flumin. 11, tab. 73. 1827, *nom. illeg.*, non Lour. 1790. TYPE.—unknown.

Polypodium pohlianum C. Presl, Tent. Pterid. 180. 1836, *nom. nud.*

Alsophila fischeriana Regel, Index Seminum Hort. Petrop. 1855, *nom. nud.*

Phegopteris scrobiculata Fée, Crypt. Vasc. Brésil 1: 102, t. 31, fig. 2. 1869. LECTOTYPE (here designated): BRAZIL. **Rio de Janeiro**: Tijuca, Bico do Papagaio, [22°56′S, 43°17′W], 12 Mar 1867, *Glaziou 2066, pro parte* (P-00610859; duplicates: C, K, MO, P, U; photos MICH, NY ex C).

Leaves to 1.0–1.5 m long; *scales of the petiole bases* ca. 1.5 × 0.05–0.07 cm long, linear, sparsely denticulate, brown, slightly twisted, en masse forming a woolly tuft; *laminae* 1 m long, to 2-pinnate at base (rarely 1-pinnate-pinnatisect), 1-pinnate-pinnatifid medially; *basal pinnae* 20–40 cm long, stalks to 1 cm long, inequilateral, pinnules acroscopically not or only slightly reduced toward pinna bases; *pinna rachises* abaxially non-glandular, glabrous to puberulent, sparsely scaly, hairs 0.1–0.2 mm long, 1–3-celled, scales 1–

2 mm long, narrowly lanceolate, brown, denticulate, flat (non-bullate), adaxially non-glandular, glabrous; *costules* abaxially non-glandular, glabrous to puberulent, hairs 0.1–0.2 mm long, 1–3-celled, scaly, scales ca. 1 mm long, linear to narrowly lanceolate, non-bullate, sparsely denticulate, adaxially glabrous; *laminar tissue between veins* abaxially non-glandular, glabrous to subglabrous, hairs (when present) ca. 0.2 mm long, 1- or 2-celled, uniseriate scales often present, these appressed, reddish, inconspicuous, adaxially non-glandular, glabrous to sparsely pubescent (often near margins), hairs 0.3–0.4 mm long, 2- or 3-celled; *veins* visible or obscure on both surfaces, non-glandular, glabrous to sparsely pubescent (similar to lamina tissue between veins); *lamina margins* sparsely to densely ciliate, hairs 0.2–0.4 mm long, 1–3-celled; *indusia* absent.

Distribution and ecology.—Brazil, primarily Atlantic rainforest from Bahia to São Paulo; 300–1000 m.

Additional Specimens Examined.—BRAZIL. **Bahia:** Castelnovo, 1836, *Blanchet 2494* (P). **Espírito Santo:** Santa Teresa, Alto de Santo Antônio, terreno do Bozza, 19°54′31″S, 40°40′28″W, 760 m, 12 Jul 2007, *Labiak et al. 4041, 4047* (NY, UPCB); Santa Teresa, trilha que sobe a encosta ao lado da entrada do Country Club, 25 Feb 1996, *Salino 2636* (BHCB, UC). **Rio de Janeiro:** Tijuca, [22°53′S, 43°13′W], 8 Oct 1867, *Glaziou 1676* (C, K, P, S); [22°56′S, 43°17′W], s.d., *Glaziou 1780* (C, P, RB-n.v.). Teresópolis, Parque Nacional da Serra dos Órgãos, 22°26′56″S, 42°59′06″W, 950 m, 13 Jan 2008, *Labiak et al. 4475* (NY, SP, UPCB). **São Paulo:** Ubatuba, Parque Estadual da Ilha Anchieta, [23°32′S, 45°03′W], 4–11 Jan 1993, *Salino 1649* (BHCB, UC). **Unknown:** *Claussen 2111* (NY, P); 1901, *Glaziou s.n.* (K).

Megalastrum grande is unique in the genus by having the pinna rachises glabrous adaxially. It is the least divided species in coastal Brazil, with laminae to 2-pinnate at the base, and usually broadly adnate segments that are slightly falcate (Fig. 5E). The hairs (when present abaxially) are generally inconspicuous, and the costal scales are sparse and linear to linear-lanceolate (Fig. 9J, M). Glandular hairs are absent from all parts of the plant.

Although Christensen (1920) included *Phegopteris scrobiculata* as a synonym of *Megalastrum connexum*, the type at K and MO seem to us to be typical *M. grande*.

10. *Megalastrum inaequale* (Kaulf. ex Link) A. R. Sm. & R. C. Moran, Amer. Fern J. 77: 127. 1987. *Polypodium inaequale* Kaulf. ex Link, Hort. Berol. 2: 107. 1833. *Ctenitis inaequale* (Kaulf. ex Link) Copel., Ann. Crypt. Phytopath. [Gen. Fil.] 5: 124. 1947. LECTOTYPE (here designated).—collected in Brazil in 1839 and cultivated in the Berlin Botanical Garden, "Hb Link," *collector unknown s.n.* (B-200059182; duplicates: BM, P). **Figs. 3B, 5F, 9A, B.**

Phegopteris marginans Fée, Crypt. Vasc. Brésil 105, t. 61, fig. 1. 1869. LECTOTYPE (here designated).—BRAZIL. **Rio de Janeiro**: Rio de Janeiro,

Tijuca, [22°56′S, 43°17′W], 18 Oct 1867, *A. Glaziou 1681* [“*1631*”] (P-00600397; duplicates: C, P).

Phegopteris fulgens Mett. ex Schenck, Hedwigia 35: 166. 1896, *nom. nud.*

Leaves to 3 m long; *scales of the petiole bases* ca. 2 × 0.15 cm, narrowly lanceolate, denticulate, brown, flat (not twisted), en masse not forming a woolly tuft; *laminae* 2 m long, to 3-pinnate-pinnatifid at base, 2-pinnate-pinnatifid medially, shiny on both surfaces, paler abaxially; *basal pinnae* 20–40 cm long, stalks to 1 cm long, inequilateral, pinnules acroscopically slightly reduced toward the pinna bases; *pinna rachises* abaxially non-glandular, pubescent, scaly, hairs 0.1–0.3 mm long, 1–3-celled, antrorsely substrigose (slightly curved toward apex), scales ca. 2.5 mm long, ovate-lanceolate to (less commonly) linear, light brown, subentire, flat to sub-bullate, adaxially non-glandular, densely pubescent, hairs ca. 0.5 mm long, 3- or 4-celled, strigose; *costules* abaxially non-glandular, pubescent, hairs ca. 0.1–0.2 mm long, 1–3-celled, scaly, scales like those of pinna rachises but more bullate, adaxially with hairs like those of costae; *laminar tissue between veins* non-glandular and glabrous on both surfaces; *veins* visible on both surfaces, non-glandular, abaxially sparsely pubescent and inconspicuously scaly, hairs ca. 0.1 mm long, 1-celled, slightly strigose, scales 0.3–0.4 mm long, uniseriate, appressed, reddish, adaxially sparsely puberulent, hairs 0.2–0.3 mm long, 1–3-celled; *lamina margins* thick, glabrous to sparsely ciliate, hairs ca. 0.1 mm long, 2-celled, strigose; *indusia* absent or appearing absent, if present easily overlooked, fugacious.

Distribution and ecology.—Endemic to coastal Brazil (Rio de Janeiro and São Paulo); 300–1200 m.

Additional Specimens Examined.—BRAZIL. **Rio de Janeiro:** Itatiaia, Lote 17, [22°29′46″S, 44°44′48″W], 1934, *Brade 14017* (MO, NY); Rio de Janeiro, Corcovado, 15 Jul 1866, *Glaziou 967* (C, K, P, S). **São Paulo:** Iguape, Serra do Itatins, [24°42′28″S, 47°47′18″W], Mar 1921, *Brade 8276* (C, HB, NY, R, S, UC, US); Raiz da Serra, [23°53′42″S, 46°46′30″W], 20 Jan 1907, *Wacket 21761* (GH, NY, UC).

Megalastrum inaequale is characterized by slightly antrorsely strigose hairs on the pinna rachises and costules abaxially and moderately dense, subbullate scales on the axes (Fig. 9A, B). The laminae are thick, slightly shiny adaxially, and paler abaxially.

11. ***Megalastrum indusiatum*** R. C. Moran, J. Prado & Labiak, ***sp. nov.*** TYPE.—BRAZIL. **Bahia**: Camacan, RPPN Serra Bonita, 10 km W de Camacan na estrada para Jacarecí, 6 km Sw na estrada para a RPPN e Torres de Transmissão, 15°23′35″S, 39°33′53″W, 750 m, 14 Apr 2007, *Matos et al. 1365* (holotype: UPCB; isotypes: CEPEC, NY). **Figs. 3B**, **5C**, **8J–O**.

A M. crenulanti *indusiis minoribus fugacibus, laminis abaxialiter squamis non bullatis secus axin, adaxialiter glabris inter venulas atque rhachidibus costis costulisque abaxialiter eglandulosis differt.*

Leaves to 2.0 m long; *scales of the petiole bases* ca. 2 cm long, linear, sparsely denticulate, light brown, twisted, en masse forming a dense woolly tuft; *laminae* 1 m long, to 4-pinnate-pinnatifid at base, 2-pinnate-pinnatisect medially; *basal pinnae* ca. 30–50 cm long, stalks to 2.5 cm long, strongly inequilateral, pinnules acroscopically slightly reduced toward pinna bases; *pinna rachises* abaxially non-glandular, slightly pubescent, hairs 0.8–1.0 mm long, 4- or 5-celled, scaly, scales ca. 5 mm long, lanceolate, subentire, flat (non-bullate), adaxially densely pubescent, hairs ca. 1 mm long, 3–6-celled, non-glandular; *costules* abaxially non-glandular, pubescent, hairs 0.3–0.4 mm long, 1–3-celled, scaly, scales ca. 1 mm long, non-bullate, subentire, adaxially pubescent, hairs 0.4–0.8 mm long, 1–3-celled, non-glandular; *laminar tissue between veins* abaxially non-glandular, pubescent, hairs ca. 0.1 mm long, 1-celled, adaxially non-glandular, hairs absent; *veins* visible on both surfaces, non-glandular abaxially, minutely scaly and pubescent abaxially, hairs 0.2–0.3 mm long, 1- or 2-celled, scales ca. 0.1–0.3 mm long, uniseriate, reddish, appressed; adaxially sparsely scaly and pubescent, hairs ca. 0.4–0.5 mm long, 1–3-celled, scales like those abaxially; *lamina margins* ciliate, hairs ca. 0.3–0.4 mm long, 1- or 2-celled; *indusia* present, circular, dark brown, pubescent, hairs 0.3–0.4 mm long, 1- or 2-celled.

Distribution and ecology.—Endemic to Bahia, Brazil; 100–800 m.

Additional Specimens Examined.—BRAZIL. **Bahia:** Almadina, Serra do Corcovado, 9,8 km ao SW de Coarací na estrada para Almadina, daí N até a Fazenda São José, 14°42′21″S, 39°36′12″W, 650–750 m, 19 Jul 2005, *Matos et al. 717* (UPCB); Ilhéus, Estrada entre Sururú e Vila Brasil, a 6–14 km de Sururú, a 12–20 km ao SE de Buerarema, [14°47′20″S, 39°39′56″W], 100 m, 10 Nov 1979, *Mori & Benton 12991* (NY).

Megalastrum indusiatum is characterized by small indusia (about the size of a single sporangial capsule), non-bullate scales on the axes, glabrous tissue adaxially between the veins, and non-glandular axes abaxially (Fig. 8J–O). The laminae typically dry dark brown adaxially. The indusia in *M. crenulans* are much larger and conspicuous, covering the sori.

12. *Megalastrum littorale* R. C. Moran, J. Prado & Labiak, ***sp. nov.*** TYPE.—BRAZIL. **Rio de Janeiro**: Parati, Parque Nacional da Serra da Bocaina, floresta Atlântica, 23°12′03″S, 44°49′43″W, 800 m, 7 Jan 2008, *Labiak et al. 4378* (holotype: UPCB; isotypes: NY, SP). **Figs. 3C, 7D, 12P–W.**

A M. canescenti *lamina abaxialiter pilis ca. 2 mm longis albidis et glandulis capitatis bi- vel tricellularis dense vestitae differt.*

Leaves to 2 m long; *scales of the petiole bases* ca. 1 cm, lanceolate, sparsely denticulate but not retrorsely so, brown to dark brown, without blackish denticulate margins; *laminae* 1–2 m long, 3-pinnate-pinnatifid at base, 2-pinnate-pinnatisect medially; *basal pinnae* ca. 50 cm long, stalks to 5 cm long, strongly inequilateral, pinnules acroscopically reduced toward

pinna bases; *pinna rachises* abaxially sparsely glandular, pubescent and sparsely scaly (sometimes apparently absent), glands ca. 0.1 mm long, hairs 1–2 mm long, 5–8-celled, scales ca. 1.5 mm, non-bullate, linear, subentire, adaxially apparently non-glandular, densely pubescent, hairs ca. 2 mm long, 7- or 8-celled; *costules* sparsely glandular abaxially, glands 0.1 mm long, pubescent abaxially, hairs 1–2 mm long, longer ones ca. 2 mm long, 5–8-celled, shorter ones ca. 1 mm long, 5- to 8-celled, sparsely scaly, scales to 1 mm long, uniseriate, appressed, brown; *laminar tissue between veins* abaxially glandular and pubescent, glands 0.1–0.2 mm long, 1- or 3-celled, capitate, whitish, abundant, hairs 1–2 mm long, 5- to 8-celled; adaxial surfaces densely pubescent, hairs 0.5–1 mm long, 4- to 6-celled, erect; *veins* visible and pubescent on both surfaces, hairs on abaxial surfaces 1–2 mm long, 5–8-celled, on adaxial surfaces hairs 0.5–1 mm long, 4–6-celled, glandular hairs sparse; *lamina margins* ciliate, hairs ca. 1–2 mm long, 5–8-celled, glandular hairs apparently absent; *indusia* absent.

Distribution and ecology.—SE Brazil (Rio de Janeiro and São Paulo); 0–800 m.

Additional Specimens Examined.—BRAZIL. **Rio de Janeiro:** Parati, Parque Nacional da Serra da Bocaina, 23°12′03″S, 44°49′43″W, 800 m, 7 Jan 2008, *Labiak et al. 4377* (NY, SP, UPCB). **São Paulo**: Ubatuba, próximo à Base Norte (Instituto Oceanográfico), Jul 1960, *Válio 112* (SP, SPF).

Megalastrum littorale is distinctive by being densely pubescent throughout with whitish hairs ca. 2 mm long (Fig. 12P–W). Also distinctive are its stalked glands with one or two basal cells below the capitate apical cell (Fig. 12T, V). It resembles *M. canescens* and can be distinguished from that species by couplet 7 of the key. This species grows near the coast, thus the specific epithet *littorale*.

13. *Megalastrum oreocharis* (Sehnem) Salino & Ponce, Darwiniana 45: 237. 2007. *Dryopteris oreocharis* Sehnem, Fl. Ilustr. Catarinense ASPI 1: 177. 1979. *Ctenitis oreocharis* (Sehnem) R. M. Bueno & R. M. Senna, Caderno Pesquisa, ser. Bot. (Sta. Cruz do Sul), 4: 11. 1992. TYPE.—BRAZIL. **Santa Catarina**: Lages, [27°49′0″S, 50°19′34″W], 950 m, 10 Jan 1951, *Sehnem 5508* (holotype: PACA; isotype: PACA). **Figs. 3C, 7B, 11A–E.**

Dryopteris connexa (Kaulf.) C. Chr. var. *minor* Legrand, Com. Bot. Mus. Historia Nat. Montevideo 2(21): 19. 1952. SYNTYPES: PARAGUAY. **Cerro Largo**: Isla Zapata, *Arechavaleta 2048* (P-n.v.). PARAGUAY. **Tacuarembó**: Gruta de los Cuervos, *D. Legrand 3325* (P-n.v.).

Leaves to 1 m long; *scales of the petiole bases* 1–2 × 0.15 cm, linear, sparsely denticulate, light brown to brown, twisted, en masse forming a dense wool; *laminae* 80 cm long, to 2-pinnate-pinnatisect at base, 2-pinnate-pinnatifid medially; *basal pinnae* ca. 15–20 cm long, stalks to 1 cm long, strongly inequilateral, pinnules acroscopically slightly reduced toward the pinna bases;

pinna rachises abaxially non-glandular, very sparsely pubescent, hairs 0.5–1.5 mm long, 4–9 celled, with a few (usually at pinna base) scales, these 2.5 mm long, linear, denticulate; adaxially pubescent, non-glandular, hairs 0.1–0.3 mm long, 1–4-celled; *costules* abaxially non-glandular, pubescent, sparsely scaly, hairs 0.7–1.5 mm long, 6–11-celled, scales 1–2 mm long, filiform to narrowly lanceolate, non-bullate, adaxially pubescent, hairs 0.6–1 mm long, 4–5-celled; *laminar tissue between veins* abaxially non-glandular, glabrous, sometimes with sparse uniseriate scales, these ca. 0.2 mm long, appressed, brown, adaxially glabrous; *veins* adaxially sparsely pubescent, visible, abaxially pubescent, hairs to 1 mm long, 4–10-celled, with sparse uniseriate, filiform scales, these ca. 0.2 mm long, appressed, brown, veins adaxially pubescent, hairs 1.5–2.0 mm long, 8–10-celled; *lamina margins* non-glandular, sparsely ciliate or apparently eciliate, hairs 0.1–0.2 mm long, 1(2)-celled; *indusia* absent.

Distribution and ecology.—S. Brazil (Paraná, Santa Catarina, Rio Grande do Sul), Paraguay, and Uraguay. 0–1000 m.

Additional Specimens Examined.—BRAZIL. **Paraná:** 19 Oct 1963, *Hatschbach 10745* (U). **Rio Grande do Sul:** Piratiny, [31°26′S, 53°06′W], 1892, *Lindman 869A* (S); Ex colonia Santo Ângelo, [28°17′S, 54°15′W], 1893, *Lindman 985A* (K, S). **Santa Catarina:** 6 May 1896, *Reineck s.n.* (P); Lages, [27°49′S, 50°19′W], 950 m, 10 Jan 1951, *Sehnem 5508* (PACA).

URUGUAY. **Tacuarembó:** Cerro Largo, Isla Zapata, [33°00′S, 56°00′W], 1877, *Arechavaleta s.n.* (P, S); Gruta de los Cuervos, [31°36′S, 53°06′W], 19 Mar 1913, *Osten 6619* (S, US).

Megalastrum oreocharis resembles a small version of *M. connexum* but differs by longer hairs (0.7–1.7 mm) on the pinna rachises, costules, and veins abaxially (Fig. 11A–E). The plants are generally thinner textured than *M. connexum*. From *M. wacketii*, it differs by the glabrous laminar tissue between the veins. Geographically, *M. oreocharis* and *M. wacketii* do not overlap (cf. Fig. 3C and Fig. 4A). In our region, *M. oreocharis* has the southernmost distribution of any species.

14. *Megalastrum organense* R. C. Moran, J. Prado & Labiak, ***sp. nov.*** TYPE.—BRAZIL. **Rio de Janeiro**: Mun. Teresópolis, Parque Nacional da Serra dos Órgãos, Floresta Atlântica, 22°26′56″S, 42°59′06″W, 1700 m, 13 Jan 2008, *Labiak et al. 4485* (holotype: UPCB; isotypes: NY, SP). **Figs. 3D, 7E, 8T–W.**

A M. retrorso *laminis utrinque inter venulas glabris, differt.*

Leaves to 1.5 m long; *scales of the petiole bases* ca. 1.5 × 0.15 cm, narrowly lanceolate, retrorsely denticulate, brown, flat (not twisted), en masse not forming a woolly tuft; *laminae* to 1 m long, to 3-pinnate-pinnatifid at base, 2-pinnate-pinnatisect medially, dull on both surfaces, paler abaxially; *basal pinnae* 35–45 cm long, stalks to 1.5 cm long, inequilateral, pinnules

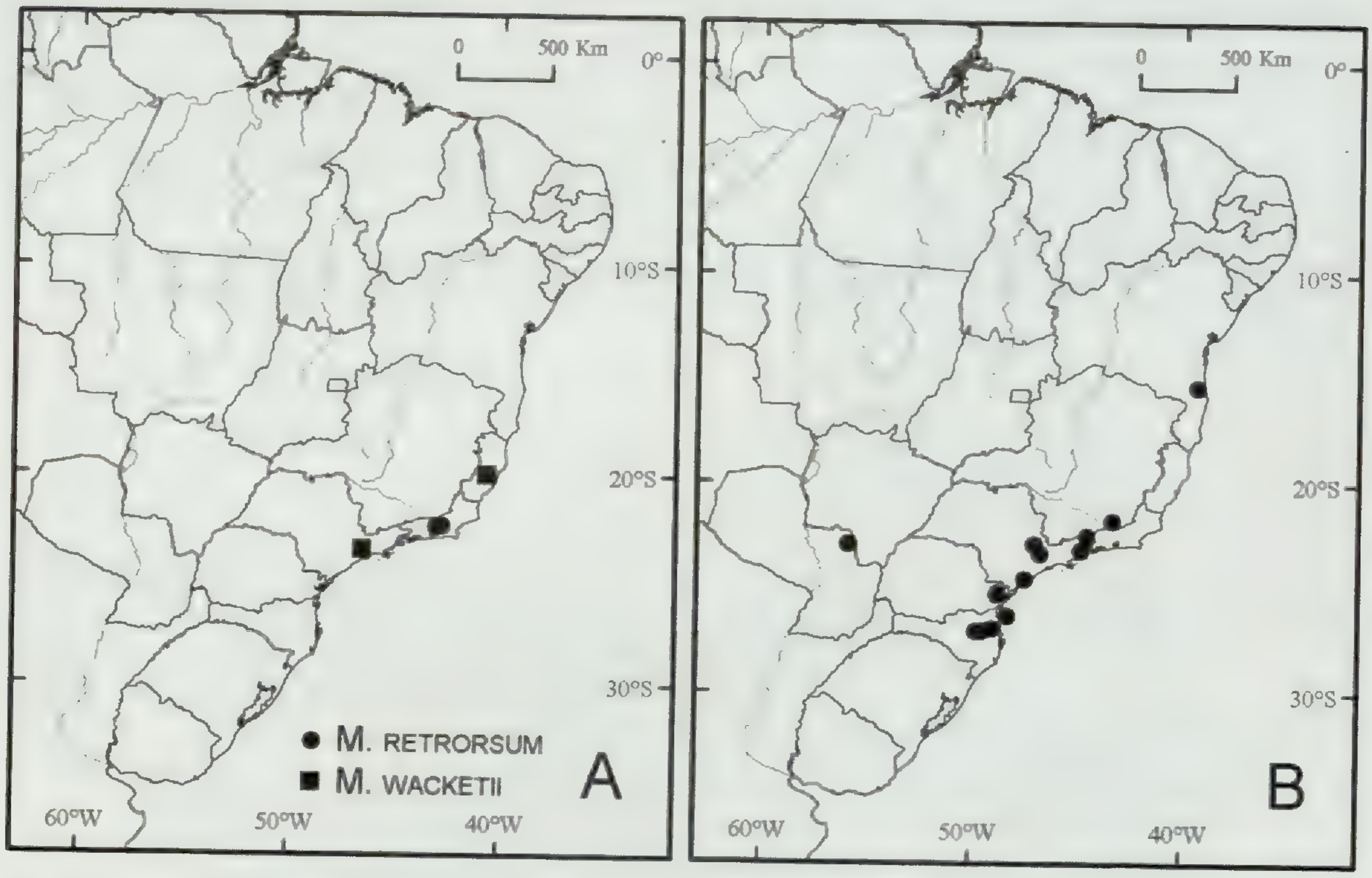

FIG. 4. Distribution of three species of *Megalastrum* in Brazil and Paraguay. A. *M. retrorsum* and *M. wacketii*. B. *M. umbrinum*.

acroscopically slightly reduced toward the pinna bases; *pinna rachises* abaxially non-glandular, pubescent to glabrescent, scaly, hairs 0.2–0.3 mm long, 3- or 4-celled, patent, scales ca. 3–7 mm long, narrowly lanceolate to linear, brown, retrorsely denticulate, flat (not bullate), adaxially non-glandular, pubescent, hairs ca. 0.5 mm long, 3–5-celled, spreading to antrorsely strigose; *costules* abaxially non-glandular, puberulent, hairs ca. 0.2–0.3 mm long, 3-celled, scaly, scales like those of pinna rachises but smaller and lanceolate, entire to retrorsely denticulate so, shiny and brown, loosely appressed, ca. 3 mm long, adaxially puberulent, hairs ca. 0.1 mm long, 2- or 3-celled, spreading; *laminar tissue between veins* abaxially non-glandular, glabrous with some inconspicuous, uniseriate, appressed, reddish scales, adaxially glabrous; *veins* visible on both surfaces, non-glandular, abaxially glabrous or sparsely pubescent, hairs ca. 0.1–0.2 mm long, 1- or 2-celled, adaxially pubescent, hairs ca. 0.3–0.5 mm long, 1–3–celled, spreading to antrorsely strigose; *lamina margins* sparsely ciliate, hairs ca. 0.1–0.2 mm long, 2-celled, spreading to appressed; *indusia* absent.

Distribution and ecology.—Endemic to Rio de Janeiro, Brazil; 1500–1750 m.

ADDITIONAL SPECIMENS EXAMINED.—BRAZIL. **Rio de Janeiro:** Teresópolis, Rio Roncador, [22°24′44″S, 42°42′56″W], 1750 m, 3 Nov 1959, *Brade 9851* (BM, GH, NY, US); Teresópolis, Parque Nacional da Serra dos Órgãos, 22°26′56″S; 42°59′06″W, 1700 m, 13 Jan 2008, *Labiak et al. 4483, 4502* (NY, SP, UPCB).

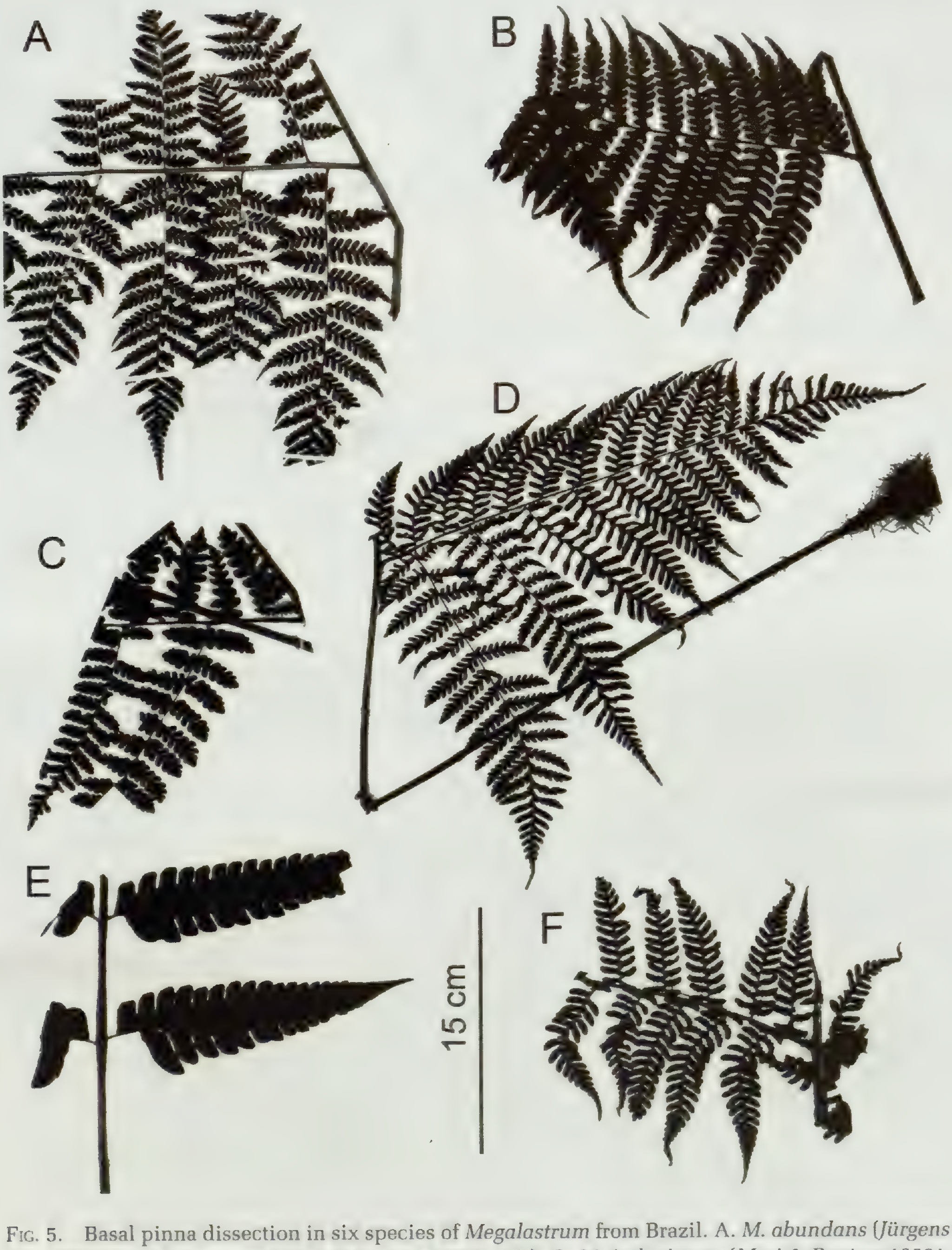

FIG. 5. Basal pinna dissection in six species of *Megalastrum* from Brazil. A. *M. abundans* (*Jürgens 195*, NY). B. *M. canescens* (*Mori & Santos 11703*, NY). C. *M. indusiatum* (*Mori & Benton 12991*, NY). D. *M. crenulans* (*Handro 2224*, US). E. *M. grande* (*Labiak et al. 4047*, NY). F. *M. inaequale* (*Luetzelburg 6911*, NY).

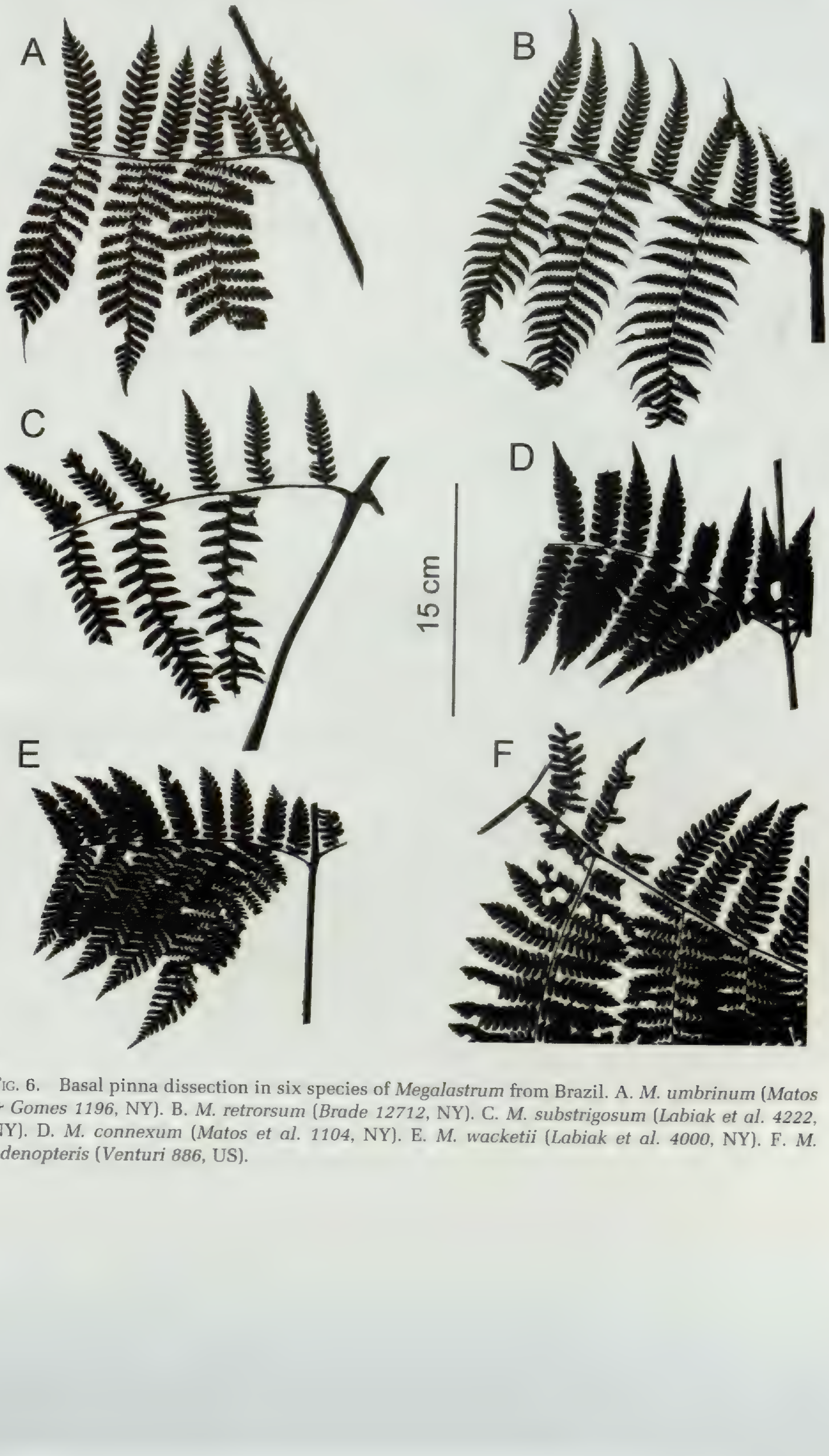

FIG. 6. Basal pinna dissection in six species of *Megalastrum* from Brazil. A. *M. umbrinum* (*Matos & Gomes 1196*, NY). B. *M. retrorsum* (*Brade 12712*, NY). C. *M. substrigosum* (*Labiak et al. 4222*, NY). D. *M. connexum* (*Matos et al. 1104*, NY). E. *M. wacketii* (*Labiak et al. 4000*, NY). F. *M. adenopteris* (*Venturi 886*, US).

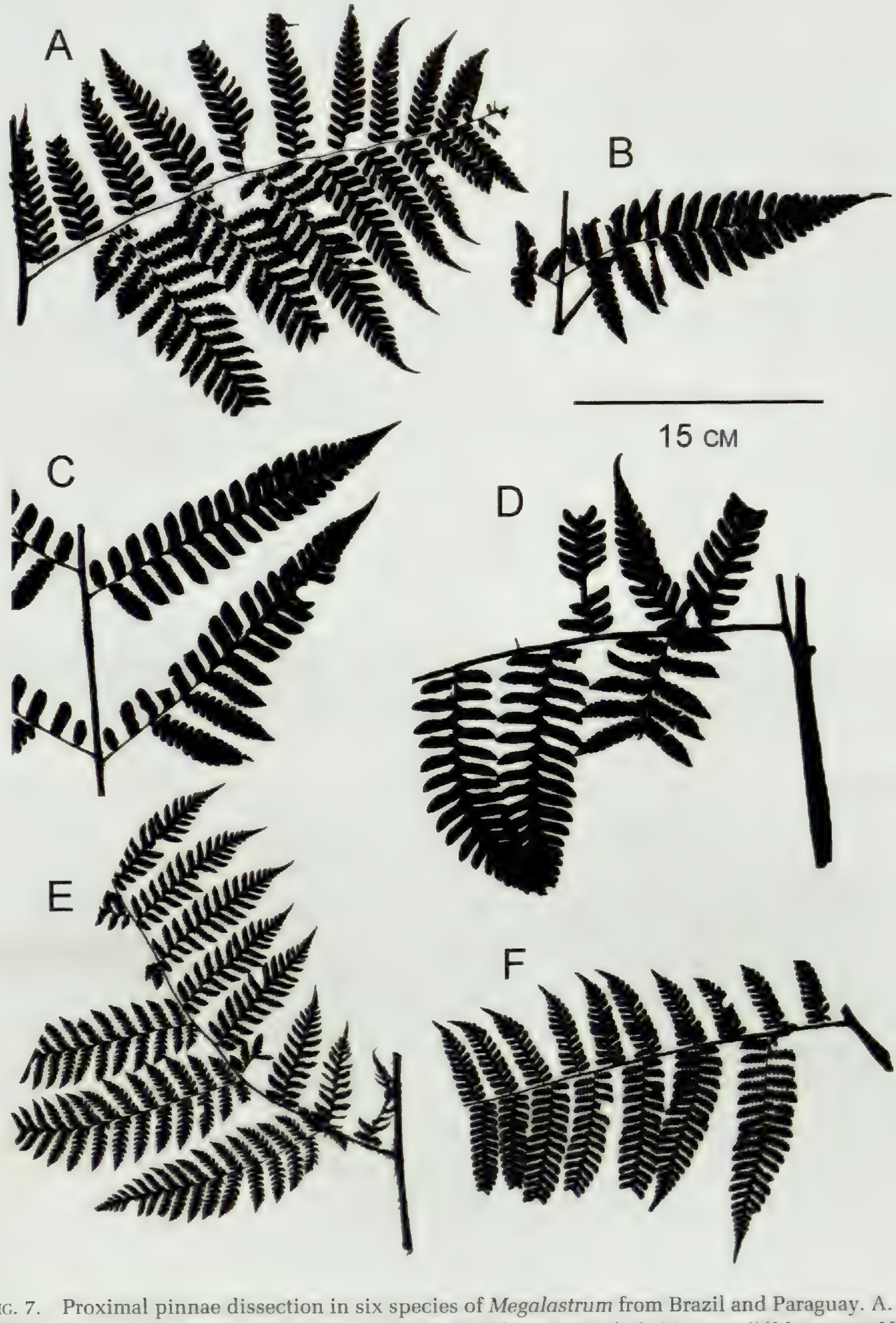

FIG. 7. Proximal pinnae dissection in six species of *Megalastrum* from Brazil and Paraguay. A. *M. albidum* (*Labiak et al. 841*, NY). B. *M. oreocharis* (*Lindman 985*, K). C. *M. eugenii* (*Matos 982*, NY). D. *M. littorale* (*Labiak et al. 4378*, NY). E. *M. organense* (*Labiak et al. 4485*, NY). F. *M. brevipubens* (*Hassler 10802*, B).

Megalastrum organense is named for the Organ Mountains of Rio de Janeiro. Like *M. retrorsum*, it has retrorsely denticulate scales (Fig. 8W). See comments under that species for comparison.

15. *Megalastrum retrorsum* R. C. Moran, J. Prado & Labiak, ***sp. nov.*** TYPE.—BRAZIL. **Rio de Janeiro**: Itatiaia, Rio Bonito, [23°10′15″S, 44°50′08″W], 900 m, Sep 1933, *Brade 12712* (holotype: NY; isotypes: MO, RB). **Figs. 4A, 6B, 8P–S.**

A M. organensi *laminis utrinque inter venulas dense et aequaliter pubescentibus differt.*

Leaves to 1.5 m long [estimate]; *scales of the petiole bases* ca. 1.5 × 0.2 cm, narrowly lanceolate, retrorsely denticulate, brown, flat (not twisted), en masse not forming a woolly tuft; *laminae* to 1 m long, to 3-pinnate-pinnatifid at base, 2-pinnate-pinnatifid medially, dull on both surfaces, paler abaxially; *basal pinnae* 30–40 cm long, stalks to 2 cm long, inequilateral, pinnules acroscopically slightly reduced toward the pinna bases; *pinna rachises* abaxially non-glandular, puberulous, scaly, hairs 0.1–0.2 mm long, 2- or 3-celled, patent, scales ca. 5 mm long, narrowly lanceolate, brown, retrorsely denticulate, flat (not bullate), adaxially non-glandular, puberulent, hairs ca. 0.4–0.7 mm long, 3–5-celled, patent; *costules* abaxially non-glandular, puberulent, hairs ca. 0.1–0.2 mm long, 1–3-celled, scaly, scales like those of pinna rachises but smaller, ca. 3 mm long, adaxially puberulent, hairs ca. 0.3–0.5 mm long, 3- or 4-celled; *laminar tissue between veins* abaxially non-glandular, densely to sparsely puberulent, hairs ca. 0.1, 1- or 2-celled, mixed with some inconspicuous, uniseriate, appressed, reddish scales, adaxially densely puberulent, hairs 0.2–0.3 mm long, 1–3-celled, erect; *veins* visible or obscure on both surfaces, non-glandular, abaxially densely puberulent and inconspicuously scaly, hairs ca. 0.1 mm long, 1- or 2-celled, adaxially densely puberulent, hairs ca. 0.3 mm, 1–3-celled; *lamina margins* sparsely puberulent, hairs ca. 0.1–0.2 mm long, 1- or 2-celled, substrigose; *indusia* absent.

Distribution and ecology.—Endemic to Rio de Janeiro, Brazil; wet forests, 900–1450 m.

Additional Specimens Examined.—BRAZIL. **Rio de Janeiro:** Parati, Parque Nacional da Serra da Bocaina, 23°10′15″S, 44°50′08″W, 1450 m, 7 Jan 2008, *Labiak et al. 4352* (NY, SP, UPCB).

Megalastrum retrorsum is named for its retrorsely denticulate scales (Fig. 8R), a character it shares with *M. organense*. It can be distinguished from *M. organense* by the abaxial surfaces of laminae densely and evenly puberulent between the veins (Fig. 8S), whereas *M. organense* is glabrous between the veins (Fig. 8V).

16. *Megalastrum substrigosum* R. C. Moran, J. Prado & Labiak, ***sp. nov.*** TYPE.—BRAZIL. **Espírito Santo**: Mun. Castelo, Parque Estadual do

FIG. 8. Indument of four species of *Megalastrum*. A–H. *M. abundans* (*Annies 117*, MICH). A. Adaxial surface of pinnule. B. Segment apex detail. C. Hairs from adaxial surface of costule. D. Abaxial surface of pinnule. E. Scale from abaxial surface of the pinna rachis. F. Hair from the pinnae rachis. G. Bullate scale from costule. H. Bullate scales from pinna rachis. J–O. *M. indusiatum* (*Mori & Benton 12991*, NY). J. Adaxial surface of pinnule. K. Scale from pinnae rachis.

Forno Grande, afloramentos rochosos, com matas úmidas nos vales, 20°31′16″S, 41°05′50″W, 1300 m, 18 Jul 2007, *Labiak et al. 4222* (holotype: UPCB; isotype: NY). **Figs. 3D, 6C, 9C, D.**

A M. inaequali *laminis crassis non lucidis, utrinque inter venulas puberulis, ad marginem ciliatis differt.*

Leaves to 1 m long [estimate]; *scales of the petiole bases* ca. 1.5 × 0.07–0.08 cm, linear, denticulate, light brown to yellowish, flat (not twisted), en masse forming a wool-like tuft; *laminae* 0.5 m long [our estimate], to 3-pinnate-pinnatifid at base, 2-pinnate-pinnatifid medially, drying light green on both surfaces; *basal pinnae* to 35 cm long, stalks to 2 cm long, strongly inequilateral, pinnules acroscopically slightly reduced toward pinna bases; *pinna rachises* abaxially non-glandular, pubescent, scaly, hairs 0.2–0.5 mm long, 1–3-celled, scales ca. 1.3–2 mm long, narrowly lanceolate, brown, sparsely denticulate, flat (non-bullate), adaxially non-glandular, densely pubescent, hairs ca. 0.4–0.5 mm long, 2–4-celled, strigose; *costules* abaxially non-glandular, pubescent, scaly, hairs ca. 0.2–0.4 mm long, 2–3-celled, scales like those of the pinna rachises, also with reduced, uniseriate, reddish appressed scales, adaxially densely pubescent, not scaly, hairs 0.3–0.4 mm long, 2–4-celled; *laminar tissue between veins* abaxially non-glandular, sparsely puberulent, very sparsely scaly, hairs ca. 0.2 mm long, 1- or 2-celled, spreading, scales ca. 0.3 mm long, uniseriate, linear, appressed, adaxially appearing glabrous but actually very sparsely pubescent, hairs ca. 0.2 mm long, substrigose, whitish; *veins* visible on both surfaces, non-glandular, abaxially sparsely pubescent and inconspicuously scaly, hairs 0.2–0.3 mm long, 1- or 2-celled, erect, scales 0.3–0.4 mm long, uniseriate, appressed, reddish, adaxially nearly glabrous to pubescent, hairs ca. 0.2–0.3(–0.5) mm, 1–3-celled, substrigose, scales ca. 0.3–0.4 mm long, uniseriate, appressed, reddish; *lamina margins* ciliate, hairs ca. 0.2 mm long, 1- or 2-celled, substrigose; *indusia* absent.

Distribution and ecology.—Endemic to Espírito Santo, known only from the type; 1300 m.

Megalastrum substrigosum can be recognized by substrigose hairs (thus the meaning of the specific epithet) on the abaxial surfaces of the pinna rachises, costules, and veins (Fig. 9D). Also characteristic are the puberulent abaxial lamina surfaces between the veins (Fig. 9D). The substrigose hairs along the

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L. Hair from veins on abaxial lamina surface. M. Abaxial surface of pinnule. N. Hair from abaxial surface of costule. O. Indusium. P–S. *M. retrorsum* (*Brade 12712*, NY). P. Adaxial surface of pinnule. Q. Hair from adaxial surface of lamina. R. Scale from abaxial surface of pinnae rachis. S. Abaxial surface of pinnule. T–W. *M. organense* (*Labiak et al. 4483*, NY). T. Adaxial surface of pinnule. U. Hair from adaxial surface of lamina. V. Abaxial surface of pinnule. W. Scale from abaxial surface of pinnae rachis. **Scale bars = 1 mm.**

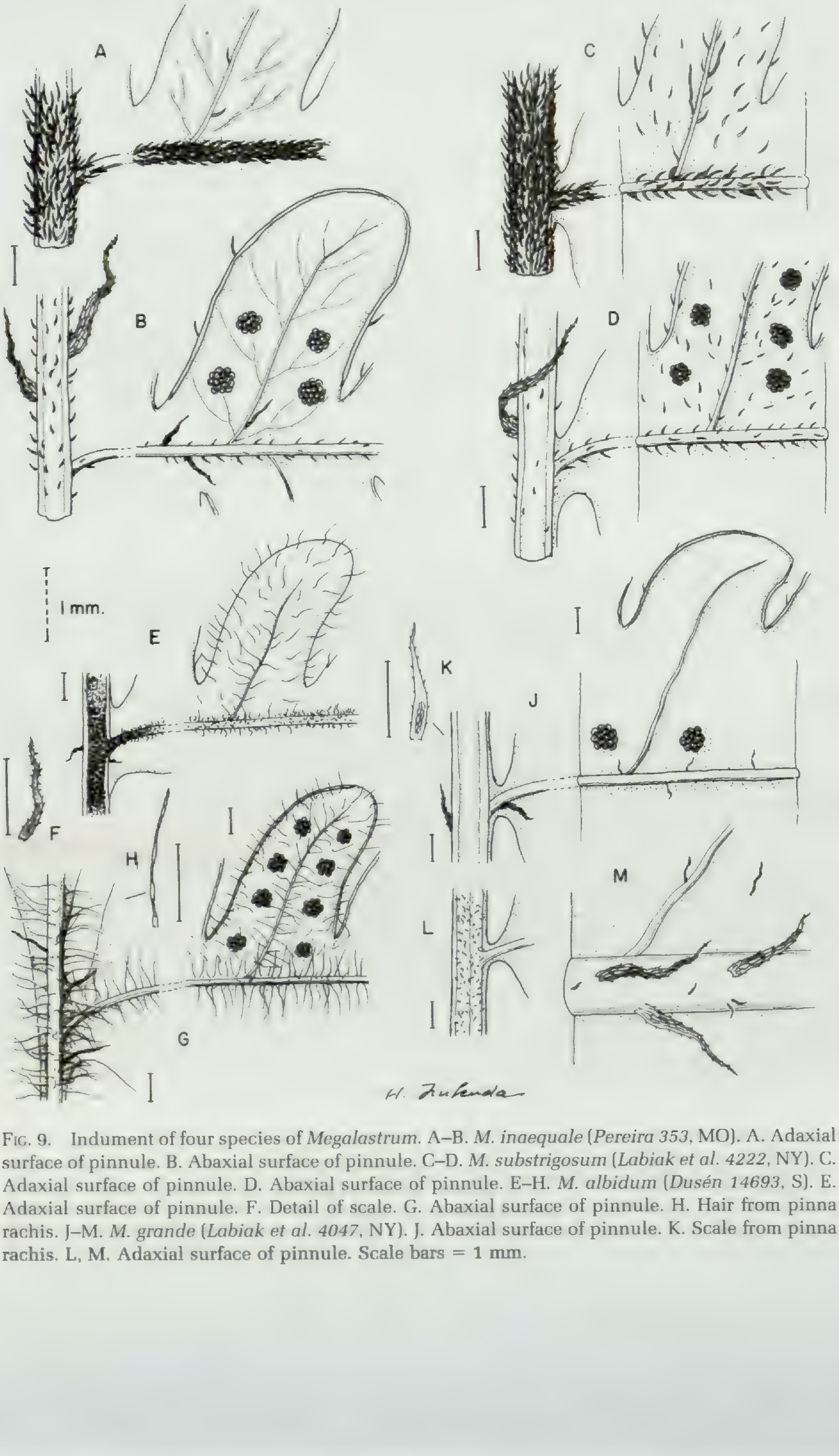

FIG. 9. Indument of four species of *Megalastrum*. A–B. *M. inaequale* (*Pereira 353*, MO). A. Adaxial surface of pinnule. B. Abaxial surface of pinnule. C–D. *M. substrigosum* (*Labiak et al. 4222*, NY). C. Adaxial surface of pinnule. D. Abaxial surface of pinnule. E–H. *M. albidum* (*Dusén 14693*, S). E. Adaxial surface of pinnule. F. Detail of scale. G. Abaxial surface of pinnule. H. Hair from pinna rachis. J–M. *M. grande* (*Labiak et al. 4047*, NY). J. Abaxial surface of pinnule. K. Scale from pinna rachis. L, M. Adaxial surface of pinnule. Scale bars = 1 mm.

axes are exactly like those of *M. inaequale*. That species, however, differs by laminae glabrous between the veins on both surfaces, thick shiny laminae, and glabrous to sparsely ciliate lamina margins (Fig. 9A, B).

17. *Megalastrum umbrinum* (C. Chr.) A. R. Sm. & R. C. Moran, Amer. Fern J. 77: 129. 1987. *Dryopteris umbrina* C. Chr., Kongel. Danske Vidensk. Selsk. Skr., Naturvidensk. Math. Afd., ser. 8, 6: 81. 1920. *Ctenitis umbrina* (C. Chr.) Ching, Sunyatsenia 5: 250. 1940. LECTOTYPE (here designated).—Brazil. **São Paulo**: Cantareira [ca. 23°20′S, 46°41′W], Jun 1913, *Brade 6532* (NY-00149429; duplicates: BM-n.v., R-n.v.; photo MICH ex BM). **Figs. 4B**, **6A**, **10A–F**.

Leaves to 1.5 m long; *scales of the petiole bases* 1.5–2 × 0.07–0.1 cm, linear, sparsely denticulate (nearly entire), brown; *laminae* ca. 1 m long, 3-pinnate-pinnatisect at base, 2-pinnate-pinnatisect medially; *basal pinnae* ca. 30 cm long, strongly inequilateral, pinnules acroscopically reduced toward pinna bases; *pinna rachises* abaxially glandular, pubescent, scaly, glands ca. 0.05 mm long, sessile, yellowish, hairs of two sizes, from 0.1–0.4 mm long, 2–6-celled, acicular, scales to 3.5 mm long, lanceolate, non-bullate, entire to subentire, brown, spreading, adaxially inconspicuously glandular, pubescent, glands like those abaxially, hairs 0.4–0.7 mm long, 2- to 5-celled; *costules* abaxially with indument like that of the pinna rachises, adaxially pubescent, not scaly, hairs 0.4–0.7 mm long, 2–5-celled, erect; *laminar tissue between veins* abaxially sparsely glandular, not scaly, hairs ca. 0.1–0.2 mm long, 1- or 2-celled, erect to (rarely) appressed, inconspicuous, adaxially glabrous or rarely with a few hairs near margins; *veins* visible on both surfaces, pubescent on both surfaces, abaxially hairs 0.2–0.3 mm long, 1- or 2-celled, adaxially the hairs denser, 0.1–0.5 mm long, 2–5-celled, spreading to appressed; *lamina margins* ciliate, non-glandular, hairs 0.2–0.3 mm long, 2- or 3-celled, appressed; *indusia* absent.

Distribution and ecology.—Southeastern Brazil, Paraguay; 100–1200 m.

Additional Specimens Examined.—BRAZIL. **Bahia:** Camacan: RPPN Serra Bonita, 9.7 km de Camacan, estrada para Jacarecí, daí 6 km SW na estrada para a RPPN e torre, 15°23′S, 39°33′W, 835 m, *Matos 1076* (UPCB). **Minas Gerais:** Caldas Novas, [17°44′S, 48°37′W], Dec 1854, *Lindberg 547* (K); Juiz de Fora, Poço Danta, [21°45′S; 43°21′W], [700] m, Jul 1902, *Schwacke 14955* (P). **Paraná:** Morretes, Estrada da graciosa, Grota Funda, [25°14′S, 48°48′W], 19 Oct 1963, *Hatschbach 10745* (HB); Antonina, Reseva Natural do Rio Cachoeira - SPVS, Trilha do meio, Floresta Ombrófila Densa, 25°18′S, 48°48′W, 200 m, 28 May 2006, *Matos & Gomes 1193* (NY, UPCB). **Rio de Janeiro:** Serra do Itatiaia, [22°29′S; 44°33′W], 900 m, 16 Jun 1930, *Brade 10056* (BM, C); Parati, Parque Nacional da Serra da Bocaina, 23°11′22″S, 44°50′15″W, 1200 m, 7 Jan 2008, *Labiak et al. 4371* (NY, SP, UPCB). **Santa Catarina:** Hansa, Blumenau, [26°56′S; 49°03′W], 1906, *Lüderwaldt s.n.* (S, US); Joinville, [26°19′S, 48°50′W], 4 Jan 1906, *Müller 162* (UC, S). **São Paulo:** São José do Barreiro,

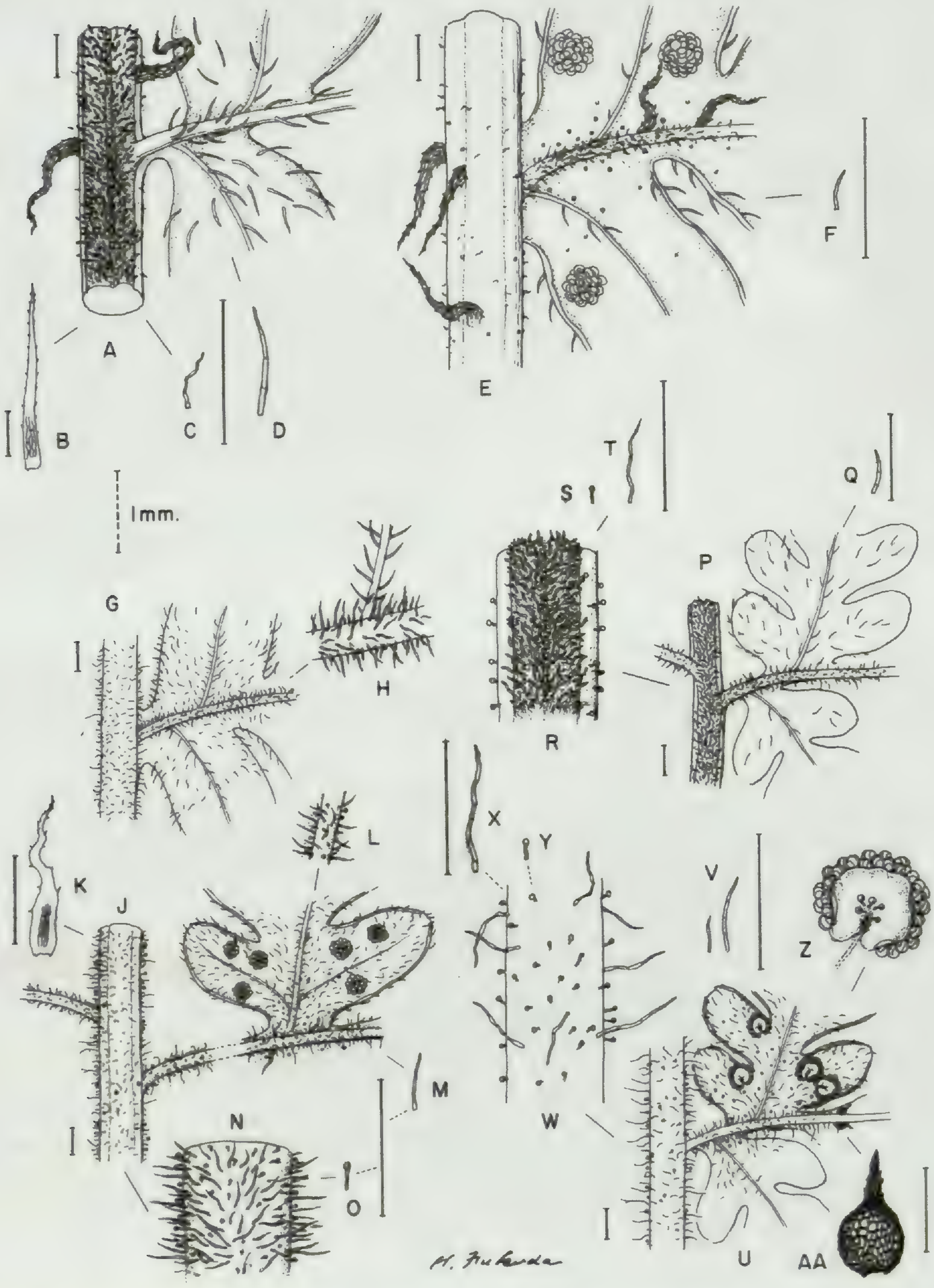

FIG. 10. Indument of four species of *Megalastrum*. A–F. *M. umbrinum* (*Brade 6532*, NY). A. Adaxial surface of pinnule. B. Detail of scale. C. Hair. D. Hair from vein adaxially. E. Abaxial surface of pinnule. F. Hair from lamina margin. G–O. *M. adenopteris* (*Jürgens s.n.* [Rosenstock Filices Austrobrasilienses no. 207], S). G. Adaxial surface of pinnule. H. Detail of costule and midrib of ultimate segment. J. Abaxial surface of pinnule. K. Scale from abaxial surface of costule.

Estrada para o Parque Nacional da Serra da Bocaina, Rodovia SP 221, 1000 m, 6 Jan 2008, *Labiak et al. 4316* (NY, SP).

PARAGUAY. **Amambay:** In altapanitie et declivibus, Sierra de Amambay, [22°56′S, 55°55′W], 1907–1908, *Hassler 10421* (BM, K, MICH).

Megalastrum umbrinum is distinctive by small but conspicuous, spreading scales along the rachises and pinna rachises—a characteristic that will help distinguish this species from many others in Brazil (Fig. 10E). Also helpful are the abaxial surfaces of the axes that are densely puberulent with short glandular hairs, and acicular (non-glandular) hairs of mixed sizes (Fig. 10E).

The most similar species is *Megalastrum adenopteris*, which differs by the adaxial surfaces between the veins densely and evenly pubescent (Fig. 10G). It also has minute fugacious indusia.

18. *Megalastrum wacketii* (Rosenst. ex C. Chr.) A. R. Sm. & R. C. Moran, Amer. Fern J. 77: 129. 1987. *Dryopteris wacketii* Rosenst. ex C. Chr., Kongel. Danske Vidensk. Selsk. Skr., Naturvidensk. Math. Afd., ser. 8, 6: 84. 1920. *Ctenitis wacketii* (Rosenst. ex C. Chr.) Ching, Sunyatsenia 5: 250. 1940. TYPE.—Brazil. **São Paulo**: Pilar, *Wacket 223* (holotype: BM). **Fig. 4A, 6E, 12G–O.**

Leaves to 1.5 m long [estimate]; *scales of the petiole bases* ca. 2 × 0.3–0.35 cm, narrowly lanceolate to linear, sparsely denticulate, brown, flat (not twisted), en masse not forming a woolly tuft; *laminae* 1 m long [our estimate], to 3-pinnate-pinnatisect at base, 2-pinnate-pinnatifid medially; *basal pinnae* to 35 cm long, stalks to 1 cm long, strongly inequilateral, pinnules acroscopically slightly reduced toward pinna bases; *pinna rachises* abaxially non-glandular, moderately to densely pubescent, very sparsely scaly, hairs 0.3–1.0 mm long, 2–7-celled, scales 0.5–1 mm long, narrowly lanceolate to linear, brown, entire to sparsely denticulate, flat (not bullate) adaxially non-glandular, densely pubescent, hairs ca. 1 mm long, 5- or 6-celled, patent, not strigose; *costules* abaxially non-glandular, pubescent, hairs generally of two lengths, long ones ca. 0.8–1.0 mm long, 4- or 5-celled, and shorter ones ca. 0.2 mm long, 1- or 2-celled, scaly, scales like those of pinna rachises, adaxially non-glandular, pubescent, hairs of two sizes, longer ones ca. 1 mm, 3- or 4-celled, and shorter ones ca. 0.3 mm, 1- or 2-celled; *laminar tissue between veins* abaxially glandular, densely pubescent, and sparsely scaly, glands 0.1–0.2 mm long, 2-

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L. Detail of costule showing hairs and glands. M. Hair from pinna rachis. N. Hairs and stalked glands on rachis. O. Glandular hair. P-AA. *M. crenulans* (*Jürgens 206*, UC). P. Adaxial surface of pinnule. Q. Hair from adaxial surface of vein. R. Detail of pinna rachis showing hairs and glandular hairs. S. Glandular hair. T. Hair from adaxial surface of pinna rachis. U. Abaxial surface of pinnule. V. Hair from laminar tissue between veins. W. Pinna rachis with hairs and glands. X. Hair. Y. Glandular hair. Z. Sorus showing indusium with glandular hairs. AA. Bullate scale. Scale bars = 1 mm.

FIG. 11. Indument of four species of *Megalastrum*. A–E. *M. oreocharis* (*Lindman A869*, S). A. Adaxial surface of pinnule. B. Scale from pinna rachis. C. Hairs on abaxial surface of pinna rachis, costule, and veins. D. Hair from pinna rachis. E. Abaxial surface of pinnule. F–K. *M. brevipubens* (*Salino 1987*, AAU). F. Adaxial surface of pinnule. G. Scale from pinna rachis. H. Hairs on abaxial surface of lamina. J. Abaxial surface of pinnule. K. Detail of indument. L–P. *M. connexum* (*Rohr*

celled, erect, capitate, hairs ca. 0.2 mm long, 1-celled, erect, scales ca. 0.3 mm long, uniseriate, linear, appressed, reddish, inconspicuous, adaxially sparsely pubescent, hairs 0.2–0.3 mm long, 1- or 2-celled, spreading, sparsely scaly, scales ca. 0.2–0.4 mm long, uniseriate, appressed, light reddish, surfaces shiny and darker than abaxial surfaces; *veins* visible on both surfaces, non-glandular, pubescent and scaly, hairs of two sizes, longer ones ca. 0.6 mm, 1–3-celled, and shorter ones ca. 0.2 mm, 1- or 2-celled, adaxially densely pubescent, hairs 0.6–0.7 mm long, 3–4(–5) celled, smaller ones ca. 0.2 mm long, 1- or 2-celled; *lamina margins* ciliate, non-glandular, hairs ca. 0.3 mm long, 1- or 2-celled; *indusia* absent.

Distribution and ecology.—SE Brazil (Espírito Santo and São Paulo; 500–1000 m.

Additional Specimens Examined.—BRAZIL. **Espírito Santo:** Jatiboca, [19°52′15″S, 40°40′15″W], 10 May 1946, *Brade & Apparicio 18071* (BM, GH, NY, U); Santa Teresa, Localidade de Julião, Floresta de encosta Semidecídua, abaixo de inselberg, 19°44′50″S, 40°40′16″W, 500 m, 10 Jul 2007, *Labiak et al. 4000, 4002* (NY, UPCB). **São Paulo:** Bosque da Saúde, [23°32′S, 46°38′W], 11 Jan 1914, *Brade 6635* (S).

Megalastrum wacketii has laminae that dry dark greenish or blackish. The short, capitate-glandular hairs are distinctive on the laminar surfaces abaxially (Fig. 12K–O). It most resembles *M. abundans* by the division of the laminae and the short (ca. 0.1 mm long) hairs on the laminar surfaces between the veins, but the latter species lacks glandular hairs on the abaxial surfaces of the laminae and has conspicuous bullate scales on the costae abaxially (Fig. 8A–H).

Acknowledgments

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1071, US). L. Adaxial surface of pinnule. M. Detail of hairs on adaxial surface. N. Abaxial surface of pinnule. O. Scale from costule. P. Detail of costule showing scale and hairs. Q–V. *M. eugenii* (*Labiak et al. 3678*, NY). Scale bars = 1 mm.

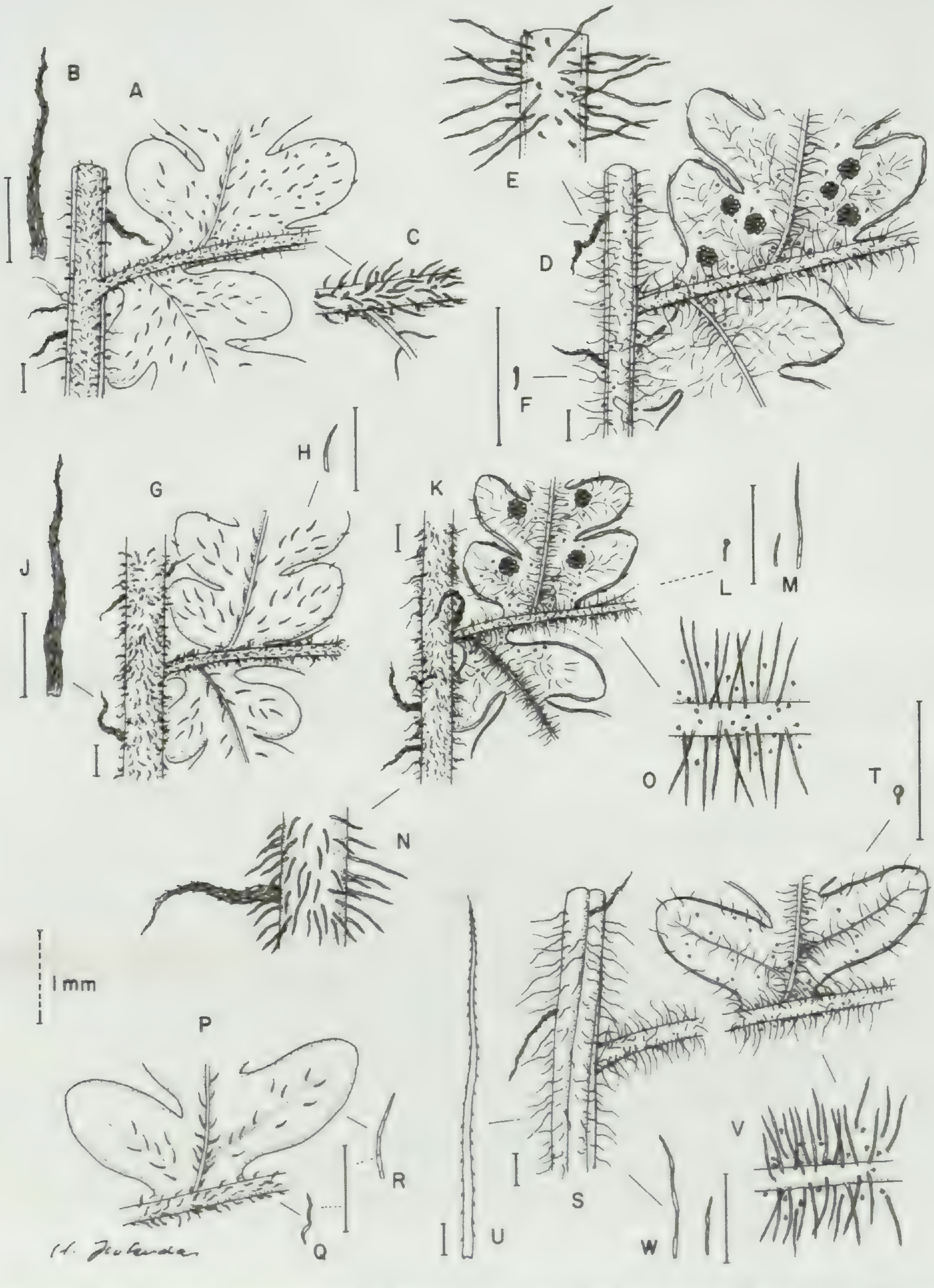

FIG. 12. Indument on four species of *Megalastrum*. A–F. *M. canescens* (*Brade 7713*, US). A. Adaxial surface of pinnules. B. Scale from rachis. C. Adaxial surface of pinna rachis showing strigose hairs and stalked glandular hairs. D. Abaxial surface of pinnule. E. Pinna rachis showing long hairs and stalked glandular hairs. F. Glandular hair. G–O. *M. wacketii* (*Labiak et al. 4002*, NY). G. Adaxial surface of pinnule. H. Hair from vein. K. Abaxial surface of pinnules. L. Glandular hair.

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M. Hairs of different sizes from pinna rachis. N. Detail of rachis showing hairs and scales. O. Detail of costules showing long spreading hairs and sessile to short-stalked glands. P–W. *M. littorale* (*Labiak et al. 4377*, NY). P. Adaxial surface of pinnules. Q. Hair from pinna rachis. R. Hair from costule. S. Abaxial surface of rachis, pinna rachis, and pinnule. T. Gland from abaxial surface of lamina between veins. U. Rachis scale. V. Costule showing hairs and glands. W. Rachis hairs. Scale bars = 1 mm.

List of Names

The numbers in parentheses refer to the species numbers assigned in the taxonomic treatment.

Alsophila fischeriana Regel (9)
Aspidium crenulans Fée (7)
Ctenitis abundans (Rosenst.) Copel. (1)
Ctenitis adenopteris (C. Chr.) Ching (2)
Ctenitis canescens (Kunze ex Mett.) C. V. Morton (5)
Ctenitis connexa (Kaulf.) Copel. (6)
Ctenitis crenulata (Fée) Ching (7)
Ctenitis eugenii (Brade) Brade (8)
Ctenitis grande (C. Presl) Copel. (9)
Ctenitis inaequale (Kaulf. ex Link) Copel. (10)
Ctenitis oreocharis (Sehnem) R. M. Bueno & R. M. Senna (13)
Ctenitis umbrina (C. Chr.) Ching (17)
Ctenitis wacketii (Rosenst. ex C. Chr.) Ching (18)
Dryopteris abundans Rosenst. (1)
Dryopteris adenopteris C. Chr. (2)
Dryopteris connexa (Kaulf.) C. Chr. (6)
Dryopteris connexa (Kaulf.) C. Chr. var. *minor* Legrand (13)
Dryopteris connexa (Kaulf.) C. Chr. var. *lateadnata* (H. Christ) C. Chr. (6)
Dryopteris crenulans (Fée) C. Chr. (7)
Dryopteris crenulans (Fée) C. Chr. f. *glandulosa* (Rosenst.) C. Chr. (7)
Dryopteris eugenii Brade (8)
Dryopteris grandis (C. Presl) C. Chr. (9)
Dryopteris lateadnata (H. Christ) C. Chr. (6)
Dryopteris martiana Rosenst. (1)
Dryopteris oreocharis Sehnem (13)
Dryopteris oreocharis Sehnem var. *canescens* Sehnem (2)
Dryopteris umbrina C. Chr. (17)
Dryopteris villosa (L.) Kuntze var. *glandulosa* Rosenst. (7)
Dryopteris villosa (L.) Kuntze var. *tomentosa* Rosenst. (2)
Dryopteris wacketii Rosenst. ex C. Chr. (18)
Dryopteris willsii (Baker) C. Chr. (6)
Nephrodium connexum (Kaulf.) Hicken (6)
Phegopteris adnata Fée (6)
Phegopteris cana Mett. (5)
Phegopteris canescens Kunze ex Mett. (5)
Phegopteris connexa (Kaulf.) Fée (6)
Phegopteris eriopoda Fée (6)
Phegopteris fulgens Mett. ex Schenck (10)
Phegopteris lateadnata H. Christ (6)
Phegopteris marginans Fée (10)
Phegopteris mollivillosa Fée (5)

Phegopteris propinqua Fée (6)
Phegopteris scrobiculata Fée (9)
Phegopteris splendida (Kaulf.) Fée (9)
Polypodium auriculatum Raddi (9)
Polypodium blanchetianum Kunze ex Mett. (5)
Polypodium connexum Kaulf. (6)
Polypodium fischerianum (Regel) Baker (9)
Polypodium formosum Raddi (9)
Polypodium grande C. Presl (9)
Polypodium inaequale Kaulf. ex Link (10)
Polypodium macropterum Kaulf. (9)
Polypodium macropterum Kaulf. var. *splendidum* Baker (9)
Polypodium pohlianum C. Presl (9)
Polypodium repandum Vell. (9)
Polypodium splendidum Kaulf. (9)
Polypodium villosum L. var. *canescens* Baker (5)
Polypodium willsii Baker (6)

List of Taxa

1. *M. abundans* (Rosenst.) A. R. Sm. & R. C. Moran
2. *M. adenopteris* (C. Chr.) A. R. Sm. & R. C. Moran
3. *M. albidum* R. C. Moran, J. Prado & Labiak
4. *M. brevipubens* R. C. Moran, J. Prado & Labiak
5. *M. canescens* (Mett. ex Kunze) A. R. Sm. & R. C. Moran
6. *M. connexum* (Kaulf.) A. R. Sm. & R. C. Moran
7. *M. crenulans* (Fée) A. R. Sm. & R. C. Moran
8. *M. eugenii* (Brade) A. R. Sm. & R. C. Moran
9. *M. grande* (C. Presl) A. R. Sm. & R. C. Moran
10. *M. inaequale* (Kaulf. ex Link) A. R. Sm. & R. C. Moran
11. *M. indusiatum* R. C. Moran, J. Prado & Labiak
12. *M. littorale* R. C. Moran, J. Prado & Labiak
13. *M. oreocharis* (Sehnem) Salino & Ponce
14. *M. organense* R. C. Moran, J. Prado & Labiak
15. *M. retrorsum* R. C. Moran, J. Prado & Labiak
16. *M. substrigosum* R. C. Moran, J. Prado & Labiak
17. *M. umbrinum* (C. Chr.) A. R. Sm. & R. C. Moran
18. *M. wacketii* (Rosenst. ex C. Chr.) A. R. Sm. & R. C. Moran

List of Exsiccatae

The numbers in parentheses refer to the species numbers assigned in the taxonomic treatment. The numbers in boldface are types.

Aguayo, A.: 216, 394 (6)
Annies, J.: 57, 65, Rosenstock Filices Austrobrasilienses no. 100 (6); **71 Rosenstock Filices Austrobrasilienses no. 117** (1)

Arechavaleta, J.: 13 (13)
Balansa, B.: 313, 2910, **313a** (6)
Basualdo, I.: 2127 (6)
Bello, W.: 533 (6)
Blanchet, J.: 1836 (6); 2454 (5); 2467, 2469, 2491 (6); 2494 (9)
Boudet-Fernandes, H. Q.: 1847 (9)
Brade, A. C.: 5373 (6); **6532** (17); 6634 (6); 6635 (18); 7713 (5); 7714 (6); 8276 (10); 8538, 8588 (9); 9691, 9695 (7); 9744 (14); 9804 (10); 9851 (14); 10056 (17); 10332 (10); **12712** (15); 14017 (10); 18071 (18); 18226 (10); 21315 (5); 21316 (6); 21444, 21445 (5); 21446 (17)
Burchell, W. J.: 1915, 1919, 1920 (6); 3160 (4)
Carauta, J. P. P.: 441, 2067 (10); 5270 (9)
Carneiro, J.: 1434 (6)
Claussen, P.: **s.n.** (6); 135, 2111 (9)
Dusén, P.: 10339, 13531, 13781 (6); 14693 (3); 15287 (6); 17563 (3); 14453a (6)
Dutra, J.: 169 (6)
Engelmann, R: RE127 (10)
Eugênio, J.: **s.n.** (8); 39, 4149 (6); 4150 (7)
Forssell, K. B. J.: 68, 240 (9)
Gardner, G.: 134 (6); 189 (7)
Gaudichaud, C.: 28 (9); 210 (10); 211 (9)
Gauthier, H.: 580 (10); 586 (9)
Gerdes, E.: 90, 90A, 91A (6)
Gillivray, J. M.: 150 (9)
Glaziou, A.: 393 (10); 966 (9); 967, 986 (10); 1676 (9); **1681** (10); **1780** (9); **1781** (7); 2066 (9); 2350, 2351 (7); 2395 (10); **2397**, **2398**, **2399** (6); 3579, 5299, 5385, 5386 (9); 7246, 7246 (6); 7676 (9); 7948 (6)
Haerchen: 79 (6)
Handro, O.: 2193 (6); 2224 (7)
Hassler, E.: 1841 (6); 6898 (7); 10278, 10420 (6); 10421 (17); **10802** (4), 12203, 12204, 12214, 12941, 12942, 12944, 12979, 12942a, 660b (6)
Hatschbach, G.: 10745 (17); 22615 (6); 24737 (3); 25584 (6); 33505 (1)
Heiner, A.: 533 (6); 601 (17)
Heinonen, S.: 6 (6); 10 (10); 33 (6)
Herter, W: 3534 (13); 4015 (9); 26004, 3534a (6); 48 (6)
Jürgens, L. C.: **s.n. Rosenstock, Filices Autrobrasilienses no. 207** (2); **s.n. Rosenstock, Filices Autrobrasilienses no. 206** (7); **195 Rosenstock, Filices Autrobrasilienses no. 367** (1);
Kegler, A.: 423 (6)
Kozera, C.: 304, 1115, 1191 (6)
Kummrow, R.: 923 (6)
Labiak, P. H. & Goldenberg, R.: **3011**, 3012 (3)
Labiak, P. H., *et al.*: 3002 (1); 3673, 3678, (8); 3716, 3721, 3762, 3852, 3903, 3931, 3983, 4260, 4262 (6); 3942 (17); 3948 (3); 3983 (6); 3984 (3); 3985 (3); 4000, 4002 (18); 4041, 4047, 4065 (9); **4222** (16); 4316 (17); 4352, 4353, 4355,

4356, 4360 (15); 4371 (17); 4372 (5); 4377, **4378** (12); 4389, 4394, 4396 (15); 4401 (10); 4403, 4407, 4425 (15); 4475 (9); 4479 (10); 4483, **4485**, 4502 (14)
Legrand, D.: **3325** (13); 3994 (6)
Leite, E.: 3567 (7); 2310 (125) (6)
Lindberg: 546 (6); 547 (17)
Lindman, C. A. M.: **s.n. (Regnell A 1313)** (2); 869A, 985A (13)
Lüderwaldt, H.: 647 (17); 648 (6); 1806 (17); 1811 (6)
Luetzelburg, P.: 6911 (10)
Luschnath: Mart. Herb. Fl. Bras. 327 (8)
Lutz, B.: 2050, 2078 (10)
Martius, P.: **320** (5); 327 (6)
Matos, F. B., *et al.*: 391 (8); 439, 613 (6); 717 (11); 978, 982 (8); 1076 (17); 1085 (6); 1094 (5); 1104, 1108 (6); 1169 (3); 1193, 1196 (17); 1326 (6); **1365** (11)
Matos, F. B. & P. Schwartsburd: 841 (3)
Mertens, K. H.: **s.n.** (9)
Mexia, Y.: 5060 (7)
Miers, J.: 189 (7)
Molas, L.: 823 (6)
Mori, S. A. *et al.*: 11515 (8); 11703 (5)
Mori, S. A. & Benton 12991 (11)
Mori, S. A. & dos Santos: 11703 (5)
Moricand, M. E.: 2469 (6)
Mosén, H.: 111 (9); 2184 (6); 2186 (17); 2187, 2188 (6); 2695 (9); 3091 (3)
Müller, O.: 76, 123 (6); 162 (17)
Mynssenn, C.: 923 (8)
Osten, C.: 6619 (13); 8435 (6)
Pabst, G.: 4542 (9); 6878 (10)
Pereira, E.: 313 (15); 353, 4133 (10)
Pietrobom, M. R.: 4394, 4698, 5225 (8)
Pohl, J. E.: **s.n.** (9)
Prado, J. & Labiak, P. H.: 1680 (6)
Raddi, J.: **s.n.** (9)
Rambo, B.: 42337, 42337 (6)
Regel, E. A.: **s.n.** (9)
Regnell, A. F.: 256 (9); 1447 (6)
Reitz, R.: 3211 (6); 12843 (1); H625 (6)
Reitz, R. & Klein, R. M.: **7516** (2)
Rick, J.: 16 (6)
Riedell, L.: 51 (5)
Rohr, J. A.: **1071** (6)
Saint-Hilaire, A. F. C. P.: 357 (9)
Saldanha, J.: 5226 (10)
Salino, A.: 1552 (6); 1649 (9); 1987 (4); 2636 (9); 30185 (6)
Sampaio, J.: 2672 (10)
Schenck: 2941 (10)
Schinini, A.: 27051 (6)

Schwacke, C. A. W.: 10230 (6); 14801 (1); 14955 (17)
Schwartsburd, P. B. & Ceolin: 1631 (1)
Schwartsburd, P. B. & Nogueira Jr.: 305 (1)
Sehnem, A.: 1343, 1346 (6); **5508** (13)
Smith, L. B.: 2227 (9); 2236 (10); 14041 (6)
Soria, N.: 2848 (4); 4082 (6)
Spannagel, C.: 254 (9); 324 (1); 481 (10); 498 (7)
Suthers, H. B.: 64a (10)
Sylvestre, L.: 1845 (15)
Tessmann, G.: 481 (17); 593 (7)
Ule, E.: 69, 193, 194, 69 p.p. (6)
Válio, I. M.: 112 (11)
Wacket, M.: 177 (10); 203 (6); **223** (18); 21761 (10)
Widgren: 372 (9)
Wills, J.: **s.n.** (6)
Zardini, E.: 2881, 11994, 40653 (6)

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Local Knowledge and Management of the Royal Fern (*Osmunda regalis* L.) in Northern Spain: Implications for Biodiversity Conservation

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Abstract.—This study reports the harvesting, management, trading and use of the royal fern (*Osmunda regalis*) in Cantabria (Spain), where medicinal plant gathering has been mainly abandoned and nowadays only few species are still commonly gathered. We interviewed 50 adults of different age, sex, and origins to obtain information on local knowledge and management practices of royal fern. *Osmunda regalis* is locally considered a highly efficient remedy. The rhizome has been traditionally employed in Cantabria mainly for the treatment of bone fractures, joint disorders and rheumatic and arthritic pain. Its consumption prevails in rural areas but it is also employed in towns and cities and its demand has led to small-scale marketing. More than half of the interviewees (54%) had only passive knowledge about their medicinal uses while the rest of informants (46%) were consumers, collectors or sellers (22% 'collector-consumers', 6% 'non collector-consumers', 4% 'collector-sellers' and 14% 'non collector-sellers'). People from villages harvested *O. regalis* for their own consumption and expressed concern about overexploitation by a rising demand from urban areas, whereas people from cities were unaware of the ecology of the fern. The scarcity of the fern has led to rural residents to develop local management practices that contribute to the species conservation. These practices included keeping the location of the fern secret, not harvesting the complete rhizome for not killing the plant and allowing its regeneration, and cultivating the species in home-gardens. The inclusion of local knowledge in harvesting regulations might result in environmental norms accepted and internalized by the local population.

Key Words.—Cantabria (Spain), ethnobotany, medicinal plants, resource management, conservation

Ferns have been employed for a wide variety of medicinal uses (e.g., Boom, 1985; Macía, 2004; Chang *et al.*, 2007) but little is known on the sustainability of fern exploitation. Monitoring and regulating wild plant harvesting can contribute to biodiversity conservation and multifunctional forest management through community-based strategies that take into account the knowledge and interests of harvesters and users (Alcorn, 1993; Berkes, 2004; Moller *et al.*, 2004; Larsen and Olsen, 2007). The proximity of customary users

to the resource facilitates their monitoring of the species. When harvesters detect signals of declining resource levels, the impact of harvesting could be regulated by decreasing the rates and quantities extracted and by limiting the areas of plant exploitation. If wild populations cannot meet harvester demand, cultivation can be the solution for avoiding overexploitation.

In developed countries, gathering wild herbs for medicinal purposes is still practiced, although is no longer a widespread practice (Rigat *et al.*, 2007). Wild plants collected for medicinal purposes include weeds (e.g., *Malva sylvestris* L.) and other common species such as *Rosmarinus officinalis* L. (Pardo-de-Santayana and Morales, 2005), and collection is mainly performed for domestic consumption. Research shows that the collection of medicinal plants in developed countries also affects some scarce plants and is also done for commercial purposes. Since the harvesting of wild medicinal plants in developed countries is not strictly regulated, the gathering and commercialization of these species are difficult to track, which hampers the ability to predict situations of overexploitation (Lange, 1998).

Here we study the harvesting of the royal fern (*Osmunda regalis* L., Osmundaceae), a subcosmopolitan fern widely distributed in some tropical and temperate regions. The royal fern is a predominantly western and southern species within Europe (Page, 1996). It grows in wetland habitats of north, middle, and west areas of the Iberian Peninsula, where it is mainly confined to riverbanks (Fig. 1a), especially in Atlantic alder groves (*Alnus glutinosa* (L.) Gaertn.) The royal fern has a long history of medicinal use (e.g., Culpeper, 1653; Austin, 2004). In the north of Spain (Galicia, Asturias, and Cantabria), the fern, locally known as *antojil*, has traditionally been employed to set broken bones, mitigate muscular ache, and treat muscle-skeletal, respiratory, and digestive disorders. Locally, the fern is prepared by maceration of the middle part of the rhizome with white wine. It is made into a bitter and mucilaginous beverage known as '*antojil* wine' (Molina, 2006) (Fig. 1b,c). *Osmunda regalis* is not listed in modern Pharmacopoeias (e.g., Real Farmacopea Española, 3rd ed. 2005; European Pharmacopoeia, 6th ed. 2007) or scientific phytotherapy books (e.g., ESCOP, 2003; Vanaclocha and Cañigueral, 2003), but Spanish regional administrations recognize that the species is gathered to be used as medicine for both domestic consumption and small-scale marketing (Gobierno de Cantabria, 2005).

In some regions of Spain (i.e., Catalonia, Basque Country, and Castilla-La Mancha), *O. regalis* is rare and is therefore catalogued as threatened (i.e., 'strictly protected', 'rare', and 'of special interest' respectively; Devesa and Ortega, 2004). In other regions, where the species has a larger range but is still not abundant (e.g., Cantabria, Asturias; Loriente, 1999), its harvesting is not regulated.

In this research, we assess individual knowledge and practices related to the medicinal use, management, and commercialization of *Osmunda regalis* in a region where its harvesting is not regulated (Cantabria, Spain). Assessing individual level variation in knowledge and practices of a wild medicinal plant can help predict future harvesting trends.

FIG. 1. a) Royal fern in his natural environment. b) Informant showing two harvested rhizomes. c) *Antojil* wine.

METHODS

We carried out five ethnobotanical fieldtrips from August 2005 to April 2006. We conducted semi-structured interviews to obtain information on local knowledge and practices regarding (1) medicinal uses (including processing and administration) and commercialization of *O. regalis* and (2) identification, habitat, and harvesting practices.

We used a quota sampling strategy to select informants of different age and sex, from rural and urban areas. People who did not know any medicinal use of the fern were not included in the study. We used snowball sampling to contact hard-to-find key informants such as sellers and gatherers (Bernard, 2006). Our final sample included 50 adults from 21 localities. The sample included men (66%) and women (34%) between 30 and 90 years of age (avg= 53 years, SD=17.14). Thirty six percent of the informants lived in localities with over 10,000 inhabitants (urban); 22% lived in villages at less than 20 km from the nearest urban settlement (nearby-rural); and 42% lived in villages farther than 20 km from the nearest urban settlement (faraway-rural).

To analyze the individual differences in knowledge and practices of *O. regalis*, we divided informants into five categories: A. Passive knowledge holder: Informants who knew at least one medicinal use of *O. regalis*, but who had never used the species; B. Non collector-consumer: Informants who had used the species, but had never collected or prepared it by themselves; C. Collector-consumer: Informants who had collected or prepared the species for domestic but not for commercial purposes; D. Collector-seller: Informants who had collected or prepared the species with commercial purposes; E. Non collector-seller: Informants who had commercialized '*antojil* wine', but had never harvested or processed the plant.

TABLE 1. Medicinal uses of *Osmunda regalis* in Cantabria. #Inf.: Number of informants who mentioned each use.

Medicinal use-category	# Inf.	Medicinal use-category	# Inf.
Muscle-skeletal System	**50**	**Others uses**	**12**
Bone regeneration	31	Tonic	6
Unspecified bone disorders	24	Digestive disorders	5
Joint or vertebral pain	13	Respiratory disorders	3
Traumatism	8	Child disorders	1
Muscular disorders	2		

RESULTS

Local knowledge.—As in other regions, in Cantabria, *antojil* has been traditionally employed for muscle-skeletal disorders (100% of the informants mentioned this use-category), including bone fractures, osteoporosis and bone decalcification (62%), joint and vertebral disorders such as rheumatic, arthritic, arthrosic or back pain (26%), traumatic injuries such as bruises, dislocations, or sprains (16%) and muscular injuries or pains (4%). The category 'unspecified bone disorders' (48%) includes muscle-skeletal conditions that were not specifically described by informants. It is also used as tonic, against rickets, digestive and respiratory disorders (Table 1). Moreover, 26% of informants reported the veterinary use of *antojil* for setting bone fractures and broken horns. Its cultural relevance is reflected in the local legend that says it can stick together a piece of meat that was previously cut in small pieces. Informants (16%) reported that they had successfully used the fern after therapies prescribed by doctors had failed. Although most doctors do not approve the use of the herbal remedy, 6% of the interviewees said that there are doctors that even recommend it for bone regeneration. Additionally a few informants (10%) reported that other healthcare professionals such as ostheopaths, medical assistants, physiotherapists and traditional healers recognize the healing virtues of *antojil*.

In Cantabria, the most usual remedy made out of *O. regalis* is *antojil* wine, which is the only remedy made out of *antojil* that is commercialized. The preparation must be drunk daily before breakfast until the patient has drunk one or two 750 ml bottles, although chronic patients take it for years. *Antojil* wine is mainly consumed by men. Its intake is not recommended for pregnant women and women of childbearing age and 18% mentioned that the remedy is considered abortive. They said that if you consume *antojil* wine "the fetus sticks to the womb", or "does not allow the womb to distend during birth". Moreover girls should not take the remedy since it is said that the "pelvis could weld before it should". Among men, *antojil* wine is mainly consumed by sportsmen and the elderly.

Osmunda regalis is consumed in rural areas but also in towns and cities. Most people in our sample that had used the remedy did not know how to prepare *antojil* wine. The demand for *antojil* wine has led to small-scale

informal marketing that originated at least 25 years ago. *Antojil* wine is commercialized through two different channels. Some people buy the remedy directly from the villagers who gather the plant and elaborate the remedy at home. The remedy can also be obtained in herbal shops or street-markets in cities. However, *antojil* wine is not a licensed product and some sellers are concerned about its marketing. In fact, two herbal shop sellers reported that they did not commercialize the remedy for this reason.

Harvesting practices.—Interviewees mentioned that *O. regalis* grows in small stream banks, rocky and steep slopes, and in north-facing places. According to informants, the rhizome from a mature specimen can measure up to 8 cm long. A new rhizome takes about 10 years to regenerate if harvesting is not destructive, i.e. cutting without uprooting the whole rhizome. Informants knew that the fern is not abundant in the region. As many as 36% of the informants associated the scarcity of the fern with bad harvesting practices and overexploitation. Several informants (12%) also reported that the abundance of the species had declined over the last decades.

In the study region, the rhizome is mainly harvested in remote zones and only by men. The rhizome is harvested during the dormant season, between November and January, using a hoe. Four harvesters reportedly know how to collect the rhizome without killing the plant. Four informants told us that it is prohibited to gather the species, although no law regulates its collection in Cantabria. Because of the perceived scarcity of the fern, rural habitants have developed local management practices that contribute to the conservation of the species. Local management practices include (1) harvesting without uprooting the whole rhizome, to allow plant restoration, (2) not sharing harvesting locations to avoid their destruction and to preserve them for later harvest, (3) harvesting from the neighboring province of Asturias where the fern is more abundant, and (4) cultivating the fern in home-gardens. Although only one informant grew the fern in his garden, eight interviewees said that they knew people that cultivated it.

Informant's distribution in the plant exploitation network.—More than half of the interviewees (54%) had only passive knowledge about the medicinal uses of *antojil*, i.e. they knew about *antojil*'s medicinal properties, but had never consumed, collected, or sold it (Fig. 2). The category of 'passive knowledge holder' included male and female informants from the three different settlement origins, mainly within the 41–50 age group (Fig. 3). Only four of the 27 'passive knowledge holders' knew how to identify the plant and could be considered potential harvesters (Table 2).

The remaining 46% of informants were involved in the plant exploitation network as consumers, harvesters, or sellers: 22% of the sample fell in the category 'collector-consumers', 4% in the category 'collector-sellers', 14% in the category 'non collector-seller', and 6% in the category 'non-collector consumers' (Fig. 2).

All the informants in the two categories of consumers ('collector-consumers' and 'non collectors-consumers') were from rural origins (Fig. 4, 5). 'Non-collector consumers' were mainly nearby-rurals ranging between 40 and 70

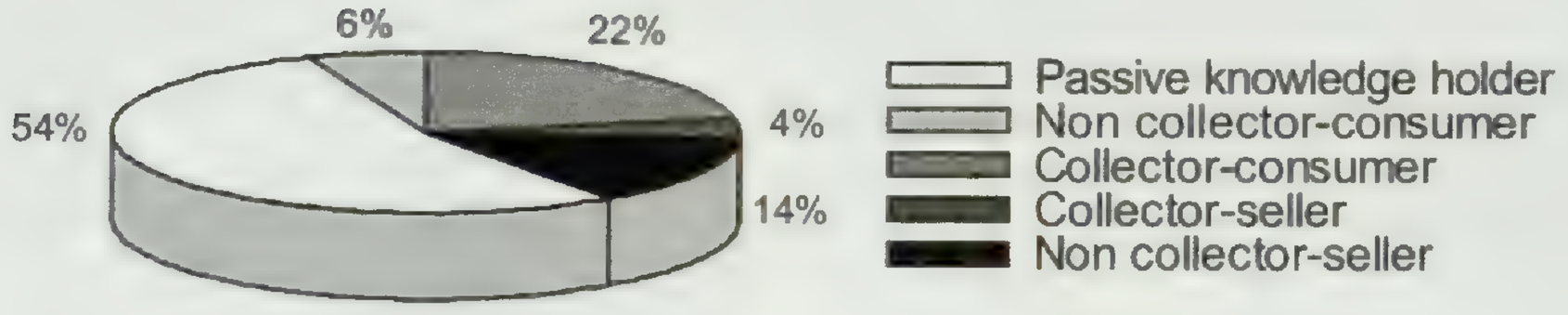

FIG. 2. Percentage of informants included in the five categories established.

years of age (avg= 55 years) whereas 'collector-consumers' were mainly faraway-rurals and elders (avg= 67 years). Collectors showed the highest knowledge about the ecological characteristics of the plant.

Sellers represented 18% of the informants and lived in urban settings (Fig. 6, 7). Fourteen percent of sellers were 'non collector-sellers', a group that included herbal shop and street-market sellers. The group included young women (Fig. 7). We only interviewed two 'collector-sellers', both restaurant owners, who had a key role in the trading circuit. 'Collector-sellers' had extensive local knowledge of the fern and a wide customer net. They even mailed the product to other provinces (Table 2).

DISCUSSION

We organize the discussion around two main topics that emerge from our findings. First, we discuss the persistence of the knowledge and medicinal use of a wild species. Second, we evaluate the distribution of harvesting and commercialization practices of *O. regalis* and its implications for conservation.

Our data suggest that *antojil* wine is still commonly known and used in Cantabria where it is considered a highly efficient remedy. We found that male and female informants from all origins and age groups reported knowing the medicinal properties of *O. regalis* (Fig. 3). The presence of young informants, especially in the categories 'passive knowledge holders' and 'non-collector

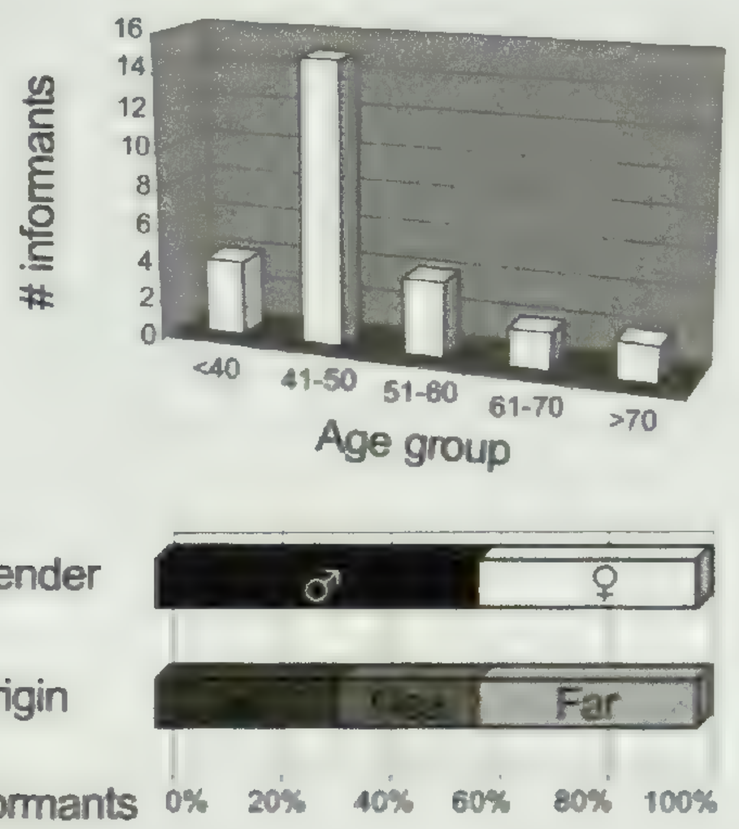

FIG. 3. Informant's distribution by sex, age group, and origin among 'passive knowledge holders'. Urb: urban; Nea: nearby-rural; Far: faraway-rural.

TABLE 2. Distribution of informants on management categories according to gender, origin, and age group.

Management categories	Gender		Urban-rural origin			Age group					Total
	male	female	urban	nearby-rural	faraway-rural	<40	41–50	51–60	61–70	>70	
Passive knowledge holder	16	11	9	7	11	4	15	4	2	2	27
Non collector-consumer	2	1	0	2	1	0	1	1	1	0	3
Collector-consumer	11	0	0	2	9	0	1	3	2	5	11
Collector-seller	2	0	2	0	0	0	1	1	0	0	2
Non collector-seller	2	5	7	0	0	4	1	1	0	1	7

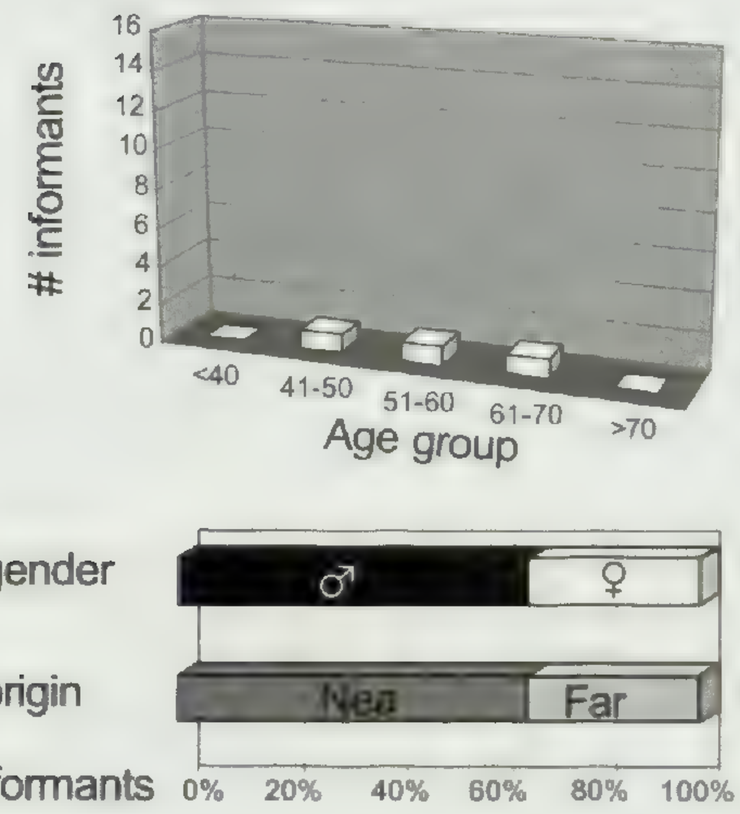

FIG. 4. Informant's distribution by sex, age group, and origin among 'non collector-consumers'. Urb, Nea, Far: see Fig. 3.

sellers' suggests that ethnobotanical knowledge of *antojil* is not threatened. Our finding meshes with previous studies in the region that describe the marketing of other medicinal plants (i.e., *Chamaemelum nobile* (L) All. and *Sideritis hyssopifolia* L.) locally preferred to pharmaceutical medicines (Pardo-de-Santayana, 2004). The results also agree with research among indigenous populations that shows that market economy is not necessarily linked to the loss of local knowledge of wild plants (e.g., Zarger and Stepp, 2004; Reyes-García *et al.*, 2007).

We found that most collectors were people over 50 years of age who lived in remote villages in direct contact with the resource (Fig. 5). Besides collectors, only four informants had the knowledge for harvesting the rhizome and preparing *antojil* wine. Our data also highlight that most sellers were young men and women living in towns and cities who lacked ecological knowledge of *antojil* (Fig. 7). Consumers were from urban and nearby-rural origins and generally also lacked the ecological knowledge of the plant. Our data suggest

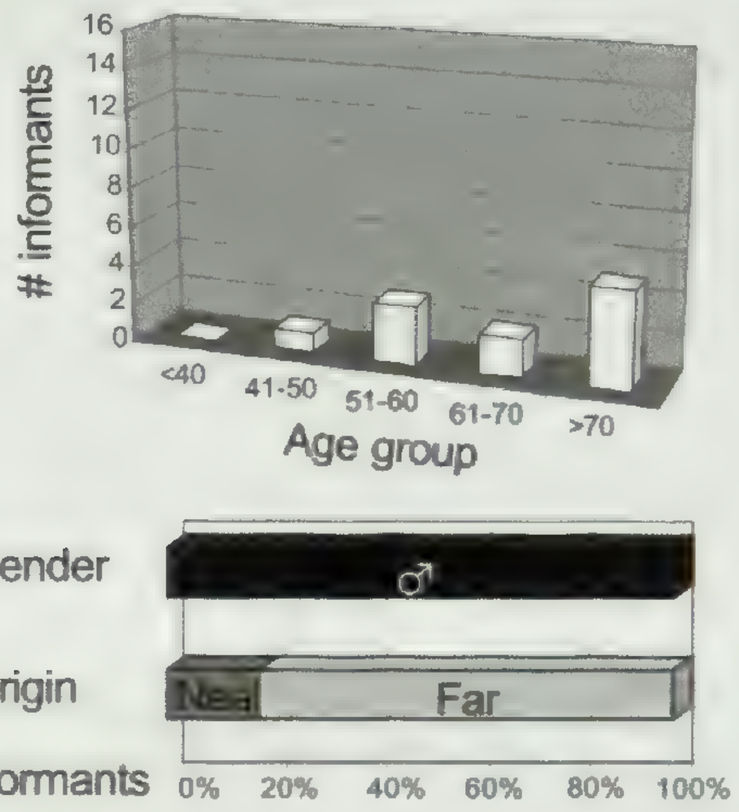

FIG. 5. Informant's distribution by sex, age group, and origin among 'collector-consumers'. Urb, Nea, Far: see Fig. 3.

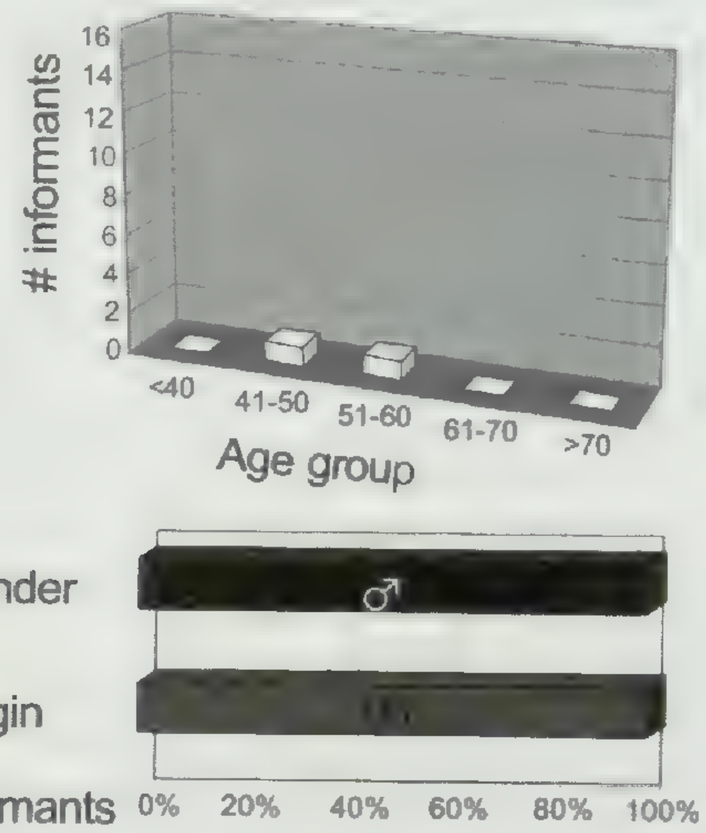

FIG. 6. Informant's distribution by sex, age group, and origin among 'collector-sellers'. Urb, Nea, Far: see Fig. 3.

that urban consumption of *antojil* might increase over the next decades due to the development of a wide urban customer network and to the presence of an aging population. The dissociation between the consumption of a remedy made from an unprotected wild plant and the ecological knowledge of the plant might have important implications for the conservation of the species. Unaware of harvesting practices, urban consumers and sellers do not understand the risk of overexploitation associated with the rising demand.

We found that all collectors expressed concern about the effects of destructive harvesting generated by a rising demand. Moreover, three 'collector consumers' expressed concern over the fact that some collectors and sellers are only driven by economic incentives and do not care about the sustainability of the resource. Informants perceive economic interests as a risk for *antojil*'s conservation and for the quality of the marketed medicinal products because some traders are diluting *antojil* wine which might destroy the efficacy of the

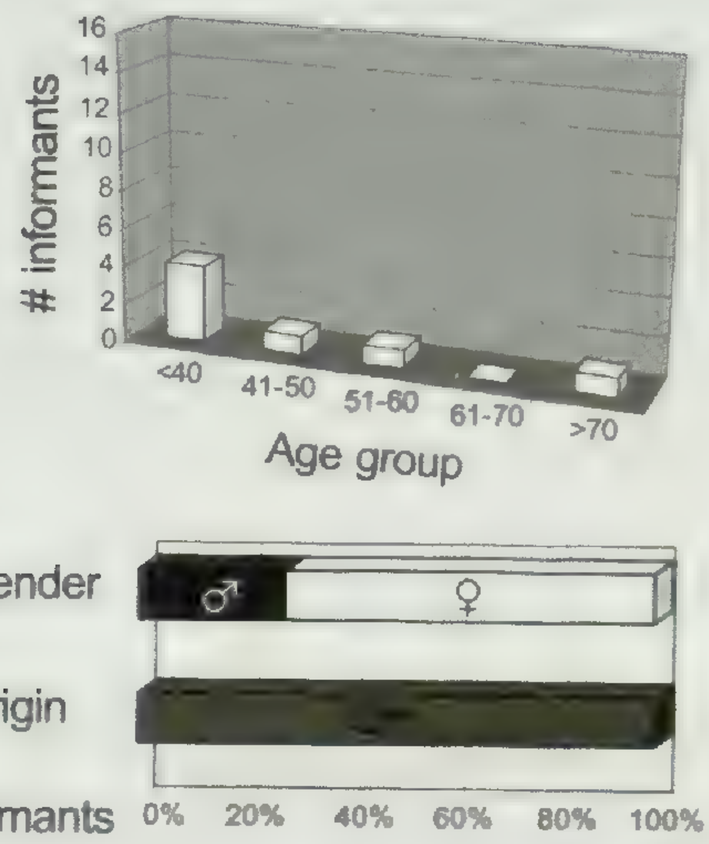

FIG. 7. Informant's distribution by sex, age group, and origin among 'non collector-sellers'. Urb, Nea, Far: see Fig. 3.

remedy. The concerns expressed by informants are similar to those reported in other case studies, when the commercialization of a medicinal plant has lead to its overexploitation (Botha *et al.*, 2004; Pardo-de-Santayana *et al.*, 2005).

Results from this research suggest that local ecological knowledge and practices are still alive in rural areas of developed countries, and that local harvesters are interested in the sustainable use of wild resources. This offers an opportunity to design management programs where local people participate actively encouraging the acceptance and internalization of environmental norms (Pardo-de-Santayana and Morales 2001).

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Shorter Notes

***Salvinia molesta* in Mexico.**—The genus *Salvinia* Ség. comprises ten species of mostly tropical ferns that are floating aquatics or less commonly stranded on receding shorelines. Among these, perhaps the best known is *S. molesta* D.S. Mitchell (Kariba weed, giant salvinia, giant water spangles), which is notorious as an extremely aggressive invasive exotic in both the New and Old Worlds. This species is extremely fast growing and has the capacity to cover the surface of even large bodies of standing and slow-moving water, forming so dense a continuous mat that oxygen exchange is inhibited and light passage is precluded, to the detriment of other aquatic organisms. Because it is a sterile pentaploid (n=45; Loyal and Grewal, Cytologia 31: 330–338. 1966), *S. molesta* reproduces only vegetatively by fragmentation and regrowth; thus humans, waterfowl, and surface drainage are the main dispersal agents. The taxon first came to the world's attention in the 1930s, when plants inadvertently released into a lake in Sri Lanka quickly grew into a major infestation. Subsequently, it caused similar problems in portions of Australia, India, southeastern Asia, and Africa (Moran, Fiddlehead Forum 19[4+5]: 26–28. 1992). It was not until 1972 that the taxon was correctly determined to represent an unnamed species and was described as new to science, based on plants infesting Lake Kariba, along the border between Zambia and Zimbabwe (Mitchell, Brit. Fern Gaz. 10: 251–252. 1972). In the United States, *S. molesta* and its relatives are considered noxious weeds under the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (USDA-APHIS) and thus are prohibited by law from international import or interstate shipment.

Salvinia molesta is a member of the *S. auriculata* Aubl. complex, which consists of four taxa, all native to South America and Trinidad (Forno, Aquatic Bot. 17: 71–83. 1983). All of these taxa (but in particular *S. molesta*) are considered potentially severe aquatic weeds outside of their native ranges. Forno and Harley (Aquatic Bot. 6: 185–187. 1979) were the first to discover native populations of *S. molesta* growing at relatively low elevations in temperate southeastern Brazil. Curiously, although the species has proliferated in the Old World tropics, it apparently has not spread significantly thus far in the neotropics (Moran, Fl. Mesoamer. 1: 396–397. 1995).

The introduction of *S. molesta* into temperate North America is very recent. Nauman (Flora of North America 2: 336–337. 1993) saw no wild-collected material, but mentioned that it was a candidate for future escape based on its cultivation in Florida. The species was first reported in the wild from a small pond in South Carolina (Johnson, Aquatics 17[4]: 22, 1985), soon thereafter from a reservoir in eastern Texas and adjacent Louisiana (Jacono, Sida 18: 927–928. 1999), and subsequently from several other southeastern states (Jacono *et al.*, Castanea 66: 214–226. 2001). At about the same time, infestations were first noted in agricultural canals in southeastern California, and subsequently in the Colorado River. *Salvinia molesta* was first reported as present on the

Arizona side of the Colorado River by Tellman (Plant Press [Tucson] 23[3]: 4, 14. 1999) and has been documented from both La Paz and Yuma Counties (Yatskievych and Windham, Canotia 4: 46é49. 2008). In California, it was first reported by Jacono and Pitman (Aquatic Nuisance Species Digest 4: 13–16. 2001). Outside the Colorado River drainage and adjacent agricultural canals in Imperial and Riverside Counties, the Consortium of California Herbaria website (http://ucjeps.berkeley.edu/consortium) now records sporadic occurrences west to San Diego County and northward to San Luis Obispo and Mendocino Counties (we have not verified these specimens).

In the recent, comprehensive account of Mexican pteridophytes (Mickel and Smith, Mem. New York Bot. Gard. 88: 1B1054. 2004), only two species of *Salvinia* were treated (*S. auriculata* and *S. minima* Baker); *S. molesta* was not mentioned. We recently were made aware of the existence of an apparent 2002 collection from the Villahermosa area in Tabasco (C. Jacono, U.S. Geological Survey, pers. comm.), which may be the earliest documented infestation of the species in the country. Given its presence in the Lower Colorado River drainage of Arizona and California, it also is not surprising that *S. molesta* should eventually become established in adjacent portions of Mexico. Anecdotal reports place the time of its first discovery in northwestern Mexico during 2003. An online document issued by the Lower Colorado River Giant Salvinia Taskforce (http://www.lcrsalvinia.org/accomplishments/Mexico-PRES%20SM%20BLYTHE%2029-JUN-05.pdf) provided photographic evidence of the presence of the species in Baja California and maps its presence nearly throughout the Mexicali Water District. Until recently, we had not seen a voucher specimen in support of these reports, but our colleague, Richard Felger, kindly collected one at our request: MEXICO, Baja California, Mpio. Mexicali, ca. 0.3 km S of Presa Morelos, wetland with stagnant water pools and sandy-silty river soils, floating aquatic, forming 100% cover on some small, stagnant pools and common at edge of large pools, 32°42′16.0″N, 114°43′44.4″W Long., elev. ca. 108 ft, 16 Mar. 2006, *R. S. Felger et al. 06-5* (BCMEX, MO, SD, UC).

Despite all of the attention given to the presence of *S. molesta* in Baja California, the species apparently has not been reported officially from the state of Sonora. However, a recently located voucher specimen was collected by the eminent Mexican aquatic plant researcher, Alejandro Novelo R.: MEXICO, Sonora, Mpio. San Luis Río Colorado, Canal de riego El Barrote, 16 km SO de San Luis Río Colorado, vegetación acuática, herbácea, hidrófita libremente flotadora de 0.10 m, asociada a *Myriophyllum* y *Potamogeton*, 7 Oct. 2004, *A. Novelo R. 4566* (MEXU).

Water that enters Mexico in the Colorado River (much of it pumped from deep wells in the desert south of Yuma, Arizona) is impounded behind the Presa Morelos, forming a reservoir that provides water for irrigation of crop fields and other human uses through a complex series of ditches and canals. The stretch of the river from below the dam to its mouth at the Gulf of California has additional water diversions. *Salvinia* can be spread by water flow through the system, as well as by human activities and waterfowl that

utilize the water and transport pieces of plant in mud attached to their feet and feathers. It seems likely that in coming years the species will continue to become increasingly common in both northeastern Baja California and northwestern Sonora. The Lower Colorado River Giant Salvinia Taskforce has completed experimental releases of the biocontrol agent for the weed, a weevil native to southern Brazil called *Cryptobagous salviniae* Calder & Sands (Coleoptera: Curculionidae), which has been used successfully in some tropical countries of the Old World (Thomas and Room, Nature 320:581–584. 1986; http://www.lcrsalvinia.org/weevil.htm). Some stands also have been sprayed seasonally with herbicides, but thus far these efforts have not succeeded in stopping the spread of *S. molesta* in the Lower Colorado River.—ARTURO MORA-OLIVO, Instituto de Ecología Aplicada, Universidad Autónoma de Tamaulipas, 13 Blvd. Adolfo López Mateos No. 928, 87040 Cd. Victoria, Tam., México, and GEORGE YATSKIEVYCH, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166 U.S.A.

Type Specimens of *Dracoglossum sinuatum* Uncovered in the Rio de Janeiro Herbarium.—During a recent survey of the A. L. A. Fée collections in the Jardim Botânico herbarium, Rio de Janeiro (RB), some specimens belonging to the newly published fern genus *Dracoglossum* Christenhusz (Thaiszia J. Bot. 17:1–10. 2007) were uncovered. These specimens are original material cited in the descriptions of *Bathmium macrocarpon* and *B. sinuatum* by Fée (Mém. Foug. 5. Gen. Filic.:288. 1852), but previously were not recognized as types. These specimens both belong to the same species: *Dracoglossum sinuatum* (Fée) Christenhusz.

Bathmium macrocarpon Fée is an illegitimate name, as stated by Christenhusz (2007), but Morton (Amer. Fern J. 56:123. 1966) erroneously took the name as a legitimate basionym and thus unintentionally established a new name: *Tectaria plantaginea* (Jacq.) Maxon var. *macrocarpa* C.V.Morton, with Fée's specimen (French Guiana, Cayenne, *Poiteau s.n.*, anno 1825) as the type. Morton assumed that this specimen was in Paris, without consulting P, but the isotypes found there are originally from the herbarium of Caen (CN), and had nothing to do with Fée. The specimen in RB is from Fée's herbarium and annotated by him and is therefore the holotype.

The specimen of *Bathmium sinuatum* was cited by Fée (1852) as "Habitat in Guyana, *Leprieur s.n.* in Herb. Moug.". The Mougeot herbarium was deposited in the herbarium of Montpellier (MPU) but this specimen is not present here. Since the specimen was considered lost, Christenhusz (2007) designated a neotype. Nevertheless the specimen in RB is original material from the Fée collection and thus it is the holotype, superseding the designated neotype.—MAARTEN J. M. CHRISTENHUSZ, Department of Biology, University of Turku, 20014 Turun yliopisto, Finland.

American Fern Journal 99(1):59–60 (2009)

Review

Illustrated Flora of Ferns and Fern Allies of South Pacific Islands, produced by the South Pacific Fern Studies Group of the Nippon Fernist Club, directed by Takehisa Nakamura and Sadamu Matsumoto. Published by the National Museum of Nature and Science as series No.8. Printed by Tokai University Press, 2008. Front matter xxxi pp., text 295 pp. Hard cover. Price: ¥ 8200 (about $80–$90 depending on exchange rate) ISBN 978-4-486-0179-2, 16 color plates; 110 black and white plates. Book 7.5 by 10.5 inches. http://www.press.tokai.ac.jp; tupcustomer@press.tokai,ac.jp; Fax: 048-432-0381.

The South Pacific islands included in this generously illustrated book are New Caledonia, Vanuatu, Fiji, Samoa, and other nearby Pacific Islands. The book is useful to botanists, students, amateurs, naturalists, and environmental workers who are interested in the flora and nature of this area. The South Pacific Fern Studies Group of the Nippon Fernist Club produced this book under the leadership of Dr. Takehisa Nakamura, former President of the Nippon Fernist Club, and Dr. Sadamu Matsumoto of the Tsukuba Botanical Garden. The Nippon Fernist Club is comprised of botanists, knowledgeable fern enthusiasts and naturalists. The fern families and their genera are treated by various well-known Japanese fern botanists.

Fortunately for those of us who do not read Japanese there are English translations throughout the book, though in some areas they are shortened versions from the Japanese text. The front matter includes maps of the area followed by colored photographs and comments on the geography of the major islands. The photographs show major island terrains and habits of some of their ferns. Following this section are several pages discussing the new classification of ferns adopted in the book. The classification system is mainly adapted from *Smith et al.* (2006*), A classification for extant ferns. Taxon 55: 705–731.*

Most of the book consists of botanical keys reaching the species level and black and white illustrations. The fern and fern allies of the area are treated family by family with each family and its contents being authored by one or more pteridologist. The family entry is followed by a brief description of the family, its possible diversification pathway, number of species, and comments on its distribution in the area. A botanical key to the genera in the family follows. Each genus is very briefly discussed and followed by a botanical key to the species of the area. Each species is then listed with reference to its diagram and coded information on the specimens collected, studied, diagramed, and the institution where vouchers are deposited. Though one might wish for lengthier descriptions, one is grateful that this work is not a mere checklist without any descriptions, botanical keys, or illustrations. The botanical keys are well constructed, clear, short and easy to use. The botanical terms used in the keys are well known and do not require a very erudite botanist to decipher. Most of the species are illustrated by excellent line

drawings, which will serve as a great help in identifying the ferns. Many of the line drawings are selected to show the diagnostic features of the species and have scale bars as well.

For English readers this book will be a valuable field guide to have when visiting these South Pacific Islands. Additionally the botanist who needs to work on ferns from this area will find careful documentation of specimens used in producing this work, a brief botanical history of the area and an extensive bibliography on South Pacific pteridophytes.

As with most bilingual books, particularly where the written languages are very different, there are typographical and other errors. However, these are relatively small distractions considering the value of having such a fern treatment on this area. We are indebt to the organizers of this team effort, Dr. Takehisa Nakamura and Dr. Sadamu Matsumoto, in producing this book and making it available to the English reading public as well.—BARBARA JOE HOSHIZAKI, 557 N. Westmoreland Ave., Los Angeles, CA 90004-2210.

INFORMATION FOR AUTHORS

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QUARTERLY JOURNAL OF THE AMERICAN FERN SOCIETY

Molecular Evidence for Genetic Heterogeneity and the Hybrid Origin of *Acrorumohra subreflexipinna* from Taiwan
Ho-Ming Chang, Wen-Liang Chiou, and Jenn-Che Wang **61**

Molecular Cloning and Sequence Analysis of Cyanovirin-N Homology Gene in *Ceratopteris thalictroides*
Xiaoqiong Qi, Yongxia Yang, Yingjuan Su, and Ting Wang **78**

A New Species of *Adiantum* from Cuba *Manuel G. Caluff* **93**

A New Brazilian Species of the Genus *Asplenium* L. (Aspleniaceae)
Fernando B. Matos, Paulo H. Labiak, and Lana S. Sylvestre **101**

New Combinations in *Pleopeltis* (Polypodiaceae) from Southeastern Brazil
Alexandre Salino **106**

A Hybrid *Phlebodium* (Polypodiaceae, Polypodiophyta) and Its Influence on the Circumscription of the Genus
J. Daniel Tejero-Díez, John T. Mickel, and Alan R. Smith **109**

2008 AFS Symposium

Summary of the 2008 AFS Symposium: From Gels to Genomics: The Evolving Landscape of Pteridology. A Celebration of Gerald Gastony's Contributions to Fern Evolutionary Biology.
Michael S. Barker and George Yatskievych **117**

A Brief History of Gerald J. Gastony's Botanical Career
Michael S. Barker and George Yatskievych **117**

Gels and Genetics: The Historical Impact of Isozymes on Paradigm Shifts in Hypotheses about Fern Evolutionary Biology
Christopher H. Haufler **125**

Using Plastid and Nuclear DNA Sequences to Redraw Generic Boundaries and Demystify Species Complexes in Cheilanthoid Ferns
Michael D. Windham, Layne Huiet, Eric Schuettpelz, Amanda L. Grusz, Carl Rothfels, James Beck, George Yatskievych, and Kathleen M. Pryer **128**

Phylogenetic Use of Inversions in Fern Chloroplast Genomes
Paul G. Wolf, Aaron M. Duffy, and Jessie M. Roper **132**

Fern Genome Structure and Evolution *Takuya Nakazato* **134**

Evolutionary Genomic Analyses of Ferns Reveal that High Chromosome Numbers are a Product of High Retention and Fewer Rounds of Polyploidy Relative to Angiosperms
Michael S. Barker **136**

Review

Flora de Nicaragua. Tomo 4. Helechos *Alan R. Smith* **142**

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Molecular Evidence for Genetic Heterogeneity and the Hybrid Origin of *Acrorumohra subreflexipinna* from Taiwan

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ABSTRACT.—*Acrorumohra subreflexipinna*, an endemic fern of Taiwan, has been suspected to be a hybrid species. The aims of this study were to detect possible multiple origins of this species, determine the genetic variation in different populations, and clarify their lineages. One nuclear and three organellar DNA fragments were sequenced to determine parentage of this putative hybrid and to examine genetic differentiation among populations. Sequence data support the conclusion that *A. subreflexipinna* arose from the hybridization of *A. hasseltii* and *A. diffracta*, and the hybridization was uni-directional, i.e., based on the assumption of maternal inheritance in organellar DNA, the former was its maternal species while the latter was its paternal source. A convincing interpretation is that the female gametes of *A. hasseltii* gametophyte could be fertilized by the male gametes from apogamous *A. diffracta*. Unique nuclear alleles present in different populations of *A. subreflexipinna* and *A. hasseltii* demonstrated that hybridization occurred many times independently. The nuclear haplotypes present in *A. subreflexipinna* were subsets of those found in the parental species, and *A. subreflexipinna* always had lower haplotype diversity than *A. hasseltii* at sympatric sites. Our results show that any genetic variation of *A. subreflexipinna* came from its parents and that it maintains this significant genetic variability because of recurrent hybridization.

KEY WORDS.—*Acrorumohra diffracta*, *Acrorumohra hasseltii*, *Acrorumohra subreflexipinna*, hybridization, monilophytes, multiple origins

Hybridization followed by polyploidization is an important mechanism driving the formation of new lineages of ferns and other plants (Paun *et al.*, 2007). By means of diploidization processes, such as chromosomal rearrangements, intergenome recombination, and gene silencing, the genomic constitution of many extant taxa might be the outcome of ancient hybridization and polyploidy (Bowers *et al.*, 2003; De Bodt *et al.*, 2005; Haufler, 1987; Paun *et al.*, 2007). Hybridization events often begin these cycles and high chromosomal base number in ferns was achieved as the result of repeated cycles of

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polyploidization (Haufler, 1987; Klekowski and Baker, 1966; Nakazato *et al.*, 2006).

Accessing the parentage of hybrids or allopolyploids is essential for understanding relationships within taxonomically complex groups. Although allozyme studies could provide tenable evidence to indicate the possible origin of hybrid-originated taxa, they have rarely been utilized to distinguish maternal lines from paternal ones. However, direct DNA evidence, such as nucleotide sequences and DNA fingerprints, can provide more informative insights into these evolutionary processes than enzymes. In most plants, organelle genomes are maternally inherited via female gametes while nuclear DNA is biparentally inherited (Soltis *et al.*, 1992). Comparing organellar DNA of hybrid taxa and their possible parents therefore could reveal the maternal origin (Gastony and Yatskievich, 1992; Vogel *et al.*, 1998) while comparing nuclear DNA of those taxa could show both putative parentages (Small *et al.*, 2004). In addition, any evolutionary trace, theoretically, would be deposited in nucleotide sequences and could be detected by DNA-based molecular technology. Unique local variation would be detectable if applicable DNA markers were chosen (Soltis *et al.*, 1992). DNA markers containing non-coding regions have been shown to be the best choice to reconstruct genealogies of hybrid and parental populations (Small *et al.*, 2004; Xiang *et al.*, 2000).

Studies of north temperate ferns have clearly indicated the contribution of hybridization and polyploidization to fern evolution (Barrington *et al*,. 1989; Bennert *et al.*, 2005; Pintér *et al.*, 2002; Wagner, 1973; Werth *et al.*, 1985). Out of the 420 species of lycophytes and ferns that grow in North America, nearly 20% are of hybrid origin (Flora of North America Editorial Committee, 1993), and reticulate networks and ploidy levels of most taxonomically complex groups have been well studied (Barrington, 1986; Stein and Barrington, 1990; Wagner, 1954, 1962, 1973; Xiang *et al.*, 2000). However, only a few ferns from other regions have received taxonomic attention like those in Europe and North America (e.g., Barrington, 1990; Ebihara *et al.*, 2005; Takamiya *et al.*, 2001; Terada and Takamiya, 2006). Some hybrid ferns have been recorded from Taiwan (Holttum and Edwards, 1986; Kuo, 1988, 1990; Miyamoto and Nakamura, 1983), but until now, no direct evidence has been reported to test and verify their parentage.

Acrorumohra is a small genus with about seven species distributed in Eastern and Southeastern Asia. This genus has an intermediate morphology between *Dryopteris* and *Arachniodes*; therefore, species of *Acrorumohra* were once treated in these genera. However, *Acrorumohra* was treated as an independent genus in the Flora of Taiwan (Shieh *et al.*, 1994) and Flora Reipublicae Popularis Sinicae (Hsieh, 2000) based on the presence of the zigzag rachis and anadromous pinnules of pinnae. *Acrorumohra subreflexipinna* (M. Ogata) H. Ito, an endemic species of Taiwan, produces shriveled and abortive spores and has an intermediate morphology between *A. hasseltii* (Blume) Ching and *A. diffracta* (Baker) H. Ito. Given its morphological characteristics, *A. subreflexipinna* has been suspected as a hybrid of these two species (Moore, 2000). Moreover, the fact that *A. subreflexipinna* always grows

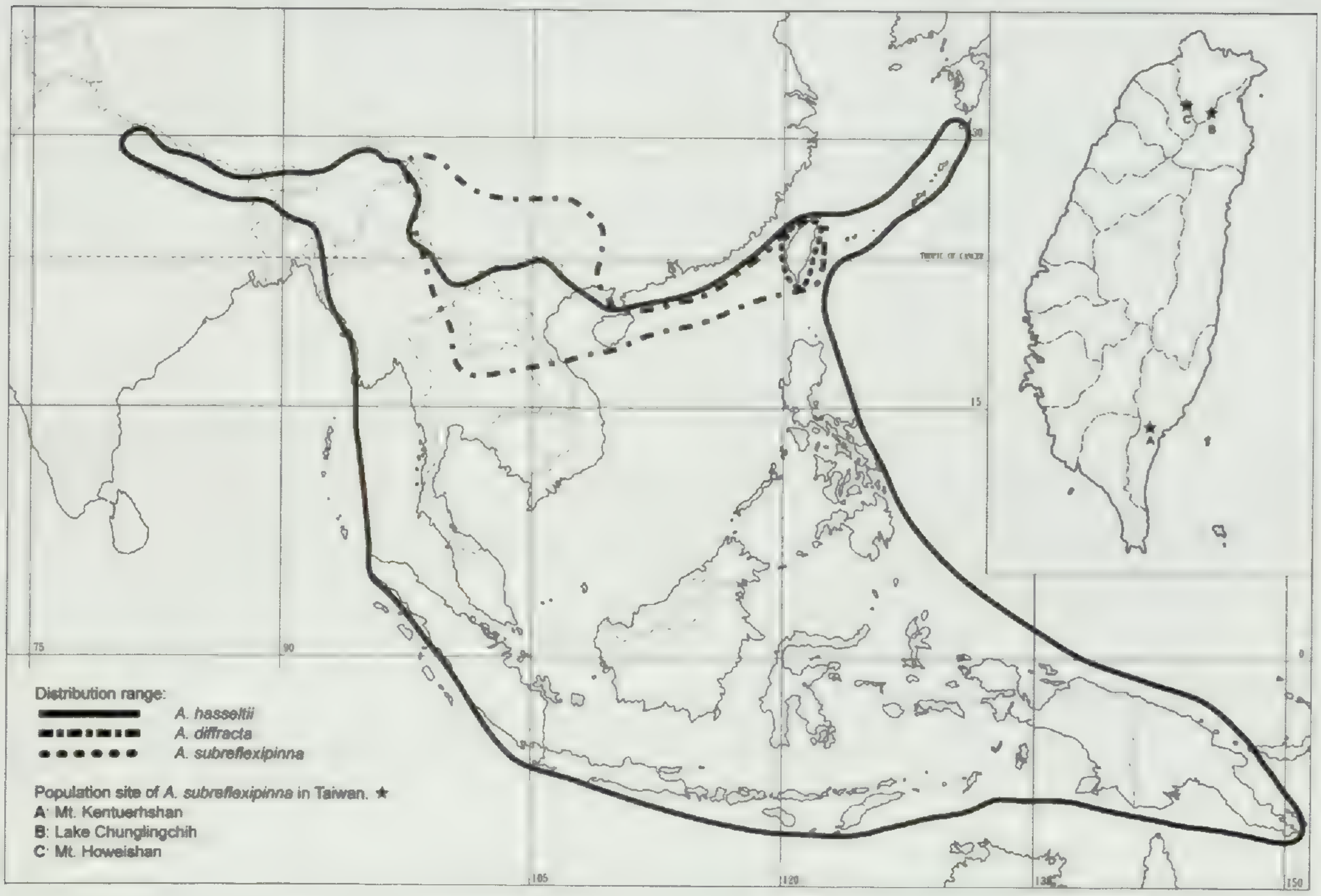

FIG. 1. Distribution of *Acrorumohra subreflexipinna*, *A. hasseltii*, and *A. diffracta*, and collection sites in this study.

sympatrically with the later two species reinforces the reasonable hypothesis of its hybrid origin. The narrowly defined genus '*Acrorumohra*' was followed and the scientific name '*A. subreflexipinna*' is used throughout the study, although palynological and unpublished breeding data indicates it a sterile F1 hybrid. In this study, chloroplast, mitochondria and nucleus DNA markers were used to identify the parentage of this suspected hybrid. Furthermore, the hypothesis that hybrid populations in Taiwan each originated independently was tested. In addition to haplotype comparison, genetic variation in different populations was determined to clarify lineage relationships.

MATERIALS AND METHODS

Plants of *Acrorumohra subreflexipinna* were sampled from three sites in Taiwan: Mt. Howeishan, Lake Chunglingchih and Mt. Kentuerhshan (Fig. 1). Leaf tissue of four to 11 individuals per population was collected for molecular analyses. Ten individuals of the two putative parent species, *A. hasseltii* and *A. diffracta*, were also sampled in each sympatric site (Table 1). Two plants of *Dryopteris polita* Rosenst. were also sampled to detect any possible parental relationship because based on phylogenetic analysis of a chloroplast *trnS-rps4* data set, *D. polita* and *A. hasseltii* are sister species (Li and Lu, 2006).

Two chloroplast intergenic spacers (*trnL-trnF* and *trnS-rps4* IGS) and one mitochondrial intron (*nad5* intron 2), which have been frequently used for

TABLE 1. Voucher information, quantity of sample and GenBank accession numbers for taxa used in molecular analysis of this study.

Taxon	Locality (population code)/sample no.	Cloned sample no./no. of clones	Voucher/deposited herbarium	DNA region/GenBank accession no.
Acrorumohra diffracta	1. Mt. Kentuerhshan (A)/10	3/15	*Chang 6316*/TNU	*trnL-trnF*/EU797681
				trnS-rps4/EU797685
				nad5 intron 2/EU797695
				pgiC intron 14–15/EU797705
	2. Lake Chunglingchih (B) /10	3/15	*Chang 6538*/TAIE	*trnL-trnF*/EU797682
				trnS-rps4/EU797686
				nad5 intron 2/EU797696
				pgiC intron 14–15/EU797706
	3. Mt. Howeishan (C) /10	3/15	*Chang 6667*/TAIE	*trnL-trnF*/EU797683
				trnS-rps4/EU797687
				nad5 intron 2/EU797697
				pgiC intron 14–15/EU797707
Acrorumohra hasseltii	1. Mt. Kentuerhshan (A) /10	3/15	*Chang 6319*/TNU	*trnL-trnF*/EU797679
				trnS-rps4/EU797691
				nad5 intron 2/EU797701
			Chang 6320/TNU	*pgiC* intron 14–15/EU797708
				pgiC intron 14–15/EU797709
	2. Lake Chunglingchih (B) /10	5/29	*Chang 6539*/TAIE	*trnL-trnF*/EU797680
				trnS-rps4/EU797692
				nad5 intron 2/EU797702
			Chang 6544/TAIE	*pgiC* intron 14–15/EU797710
			Chang 6540/TAIE	*pgiC* intron 14–15/EU797711
				pgiC intron 14–15/EU797712
	3. Mt. Howeishan (C) /10	4/23	*Chang 6673*/TAIE	*trnL-trnF*/EU797677
				trnS-rps4/EU797693
				nad5 intron 2/EU797703
			Chang 6674/TAIE	*pgiC* intron 14–15/EU797713
			Chang 6675/TAIE	*pgiC* intron 14–15/EU797714
				pgiC intron 14–15/EU797715

TABLE 1. Continued.

Taxon	Locality (population code)/sample no.	Cloned sample no./no. of clones	Voucher/deposited herbarium	DNA region/GenBank accession no.
Acrorumohra subreflexipinna	1. Mt. Kentuerhshan (A)/5	5/52	*Chang 6317*/TNU	*trnL-trnF*/EU797678 *trnS-rps4*/EU797688 *nad5* intron 2/EU797698
			Chang 6322/TNU	*pgiC* intron 14–15/EU797720 *pgiC* intron 14–15/EU797716 *pgiC* intron 14–15/EU797719
	2. Lake Chunglingchih (B)/4	4/37	*Chang 6541*/TAIE	*trnL-trnF*/EU797675 *trnS-rps4*/EU797689 *nad5* intron 2/EU797699
			Chang 6542/TAIE	*pgiC* intron 14–15/EU797717 *pgiC* intron 14–15/EU797721 *pgiC* intron 14–15/EU797723
	3. Mt. Howeishan (C)/11	11/91	*Chang 6671*/TAIE	*trnL-trnF*/EU797676 *trnS-rps4*/EU797690 *nad5* intron 2/EU797700
			Chang 6672/TAIE	*pgiC* intron 14–15/EU797718 *pgiC* intron 14–15/EU797722 *pgiC* intron 14–15/EU797724
Dryopteris polita	Lienhuachih/2	2/10	*Chang 6903*/TAIE	*trnL-trnF*/EU797684 *trnS-rps4*/EU797694 *nad5* intron 2/EU797704 *pgiC* intron 14–15/EU797725

phylogenetic analysis at lower taxonomic levels, were employed to reveal maternal history, while introns of the single-copy nuclear gene *pgiC* (including introns 14 and 15, and exon 15) were used to observe bi-parental inheritance. These sequences were chosen because of their significant phylogenetic information relative to other fragments and the availability of usable primers (Ishikawa *et al.*, 2002; Nadot *et al.*, 1995; Smith and Cranfill, 2002; Vangerow *et al.*, 1999).

Dry or fresh tissues of young leaves were homogenized with liquid nitrogen. Genomic DNA was extracted from ca. 100 mg of leaf tissue by using a Plant Genomic DNA Mini Kit (Viogene, USA). The PCR amplification of all segments was performed in an ABI thermocycler (9700). Primers for *trnL-trnF* IGS, trnLF-11 5′- GCG CAA GTT GCG GTA GAA CGA-3′ and trnLF-12 5′-CTG CTC TAC CGA CTG AG CTA-3′, were modifications of those utilized by Taberlet *et al.* (1991). The primers tsr4-f/tsr4-r 5′-CCC GCA AAG CTT AGT GAT CA-3′/ 5′-CCG AGG GTT CGA ATC CCT C-3′, nadh2-f/nadh2-r 5′-GGG GCT ATA TCG CCA TCC-3′/5′-CCG CAC GTG CAA GTT TCC-3′, and pgiC-14fA/pgiC-16rA 5′-GTG CTT CTG GGT CTT TTG AG-3′/5′-GTT GTC CAT TAG TTC CAG GT-3′ were developed for this study referring to Smith and Cranfill (2002), Vangerow *et al.* (1999) and Ishikawa *et al.* (2002), respectively. PCR reactions were carried out in 20 μL reactions containing 2 μL unstandardized template DNA, 0.2 mmol/L of each dNTP, 0.8 units of Taq polymerase (ABgene, USA) and 6.25 pmol each of the forward and reverse primers, and programmed for 5 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at annealing temperature and 2 min at 72°C, followed by a 8 min extension at 72°C. The annealing temperature was 59°C in amplifying the chloroplast *trnL-trnF* IGS and the mitochondrial *nad5* intron 2, and 52°C in amplifying the chloroplast *trnS-rps4* fragment. When amplifying nuclear *pgiC* intron 14–15 segment, annealing was performed at 57°C for the first 3 cycles, at 55°C for the next 3 and at 54°C for the final 29. PCR products were directly sequenced, using one amplification primer, on an ABI 373A automated sequencer (Applied Biosystems, USA) with the Taq Dye Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems). For the electrophoresed bands with lengths greater than 500 bp, sequences were determined in both directions. Additionally, *pgiC* intron 14–15 segments of all *A. subreflexipinna* samples and 3–5 samples in each population of *A. hasseltii*and *A. diffracta* were cloned. The PCR products of the nuclear segment were purified by electrophoresis using 1× TAE buffer on a 1.2% agarose gel. Electrophoresed bands were cut and eluted using the Gel-M gel extraction system (Viogene). Purified nuclear DNA was cloned with the yT&A cloning kit (Yeastern Biotech, Taiwan) following the manufacturer's protocol. Five to eight colonies were chosen to perform colony PCR using TA-F forward and TA-R reverse primers (Yeastern Biotech). Purified nuclear DNA was sequenced with M13 universal and reverse primers which are located on the DH5α vector termination site. When any different haplotype was detected, repeated PCR reactions using a different *Taq* polymerase (Genomics, Taiwan) or using DNA from another three colonies were chosen to check whether it was a real variant or not. All sequences were deposited in the GenBank nucleotide

sequence database, and accession numbers and their corresponding DNA regions are listed in Table 1.

The sequences were aligned by BioEdit 7.0 and manual correction, and compared with nucleotide sequences available through GenBank to determine their boundaries of coding region. Haplotypes were named after the first letter of the specific epithet, and followed by a lowercase letter and number to designate different, minor haplotypes (those differing from their corresponding major haplotype at only one base) of *A. hasseltii*. Genetic diversity at population and species levels was estimated with the software package DNA Sequence Polymorphism (DnaSP 4.20.2, Rozas *et al.*, 2003). The haplotype diversity (h) and nucleotide diversity (π) of these three populations were calculated separately and totally. Genetic differentiation (γ_{ST}, Nei, 1982) among these three populations and between pairs of populations was also calculated by this package. γ_{st}, but not F_{ST} or N_{ST}, was used because the three sampled populations were the only ones of interest (Lynch and Crease, 1990). Because no variation was detected in the nuclear sequences of *A. diffracta*, only the *A. hasseltii* haplotypes cloned from *A. subreflexipinna* were used when analyzing genetic diversity and differentiation among the populations of *A. subreflexipinna*. Haplotypes of *A. hasseltii* and *A. subreflexipinna* were identified and coded by direct sequence comparison, and unrooted haplotype networks were constructed with the program TCS 1.21 (Clement *et al.*, 2000).

Results

Total aligned length and GC content of the sequences of nuclear *pgiC* intron 14–15, chloroplast *trnL-trnF* IGS and *trnS-rps4* IGS, and mitochondrial *nad5* intron 2 were 725 bp/37.8%, 268 bp/34.9%, 374 bp/36.5%, and 728 bp/52.6%, respectively. Low GC content of chloroplast segments agreed with the AT-rich property of most non-coding spacers (Graur and Li, 2000).

In the chloroplast and mitochondria segments, all 50 individuals of *A. subreflexipinna* and *A. hasseltii* had the same nucleotide sequences but were different from those of *A. diffracta* and *D. polita* (Tables 2–4). In the nuclear *pgiC* intron 14–15 sequences, *A. subreflexipinna* possessed both the *A. hasseltii* and the *A. diffracta* haplotypes (Table 5) but not that of *D. polita* (data not shown). *pgiC* intron 14–15 sequences of *A. diffracta* from the three populations were all the same (haplotype 'D'), but those of *A. hasseltii* and *A. subreflexipinna* in each population had two to three haplotypes (Tables 5 and 6). There were two major (Ha and Hb) and another three minor haplotypes (Ha1, Hb1 and Hb2) found in *A. hasseltii* (Table 5). These minor haplotypes differ from their corresponding major haplotypes at only one base, and were found in the three populations respectively. In total, five and four haplotypes were found in *A. hasseltii* and *A. subreflexipinna*, respectively.

When calculating haplotype diversity (h) and nucleotide diversity (π) (Table 6), the haplotype "D" was, *a priori*, removed from the genetic pool of *A. subreflexipinna* to avoid interference in comparison with that of *A. hasseltii*. In *A. hasseltii*, haplotype diversity (h) among these three populations ranged

TABLE 2. The variable nucleotide sites (indel & base substitution) of chloroplast *trnL-trnF* intergenic spacer sequences. Columns shaded are sites identical to the hybrid sequences.

Species	Variable sites																	
	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	2	2
	0	1	2	2	2	2	4	6	0	4	5	5	6	8	9	9	1	3
	7	9	0	1	2	3	9	8	5	2	1	6	6	8	8	9	6	2
A. diffracta	G	-	-	-	-	-	G	A	C	T	C	A	T	C	G	C	-	C
A. subreflexipinna	A	-	-	-	-	-	A	G	T	A	C	A	C	A	A	T	-	T
A. hasseltii	A	-	-	-	-	-	A	G	T	A	C	A	C	A	A	T	-	T
D. polita	G	T	A	G	T	T	A	G	T	A	T	G	C	G	A	T	A	C

from 0.533 to 0.689, and it was 0.724 at the species level. In *A. subreflexipinna*, haplotype diversity among populations ranged from 0.400 to 0.667, and it was 0.674 at the species level. Nucleotide diversity (π) among the three populations of *A. hasseltii* ranged from 0.00074 to 0.00446, and it was 0.00485 at the species level. In *A. subreflexipinna*, nucleotide diversity among populations ranged from 0.00055 to 0.00553, and it was 0.00466 at the species level.

For the nuclear *pgiC* segment of *A. hasseltii* and *A. subreflexipinna*, the Ha haplotype could be clearly distinguished from Hb by six sites with different base pairs and two indel sites (Table 5; Fig. 2). The Ha haplotype has a minor type (Ha1) with a single base difference. This minor haplotype is found only in the Mt. Kentuerhshan population of *A. hasseltii* and *A. subreflexipinna* (Fig. 2). On the other hand, the Hb haplotype has two single base change minors (Hb1 and Hb2) occurring respectively in Lake Chunglingchih and Mt. Howeishan populations of *A. hasseltii* (Fig. 2(i)). In *A. subreflexipinna*, genetic variation among different individuals and/or populations directly came from different haplotypes of *A. hasseltii*. For example, in *A. subreflexipinna* of Mt. Kentuerhshan, except for the haplotype that was identical to *A. diffracta*, there were two haplotypes (Ha and Ha1) that were also found in *A. hasseltii* of the sympatric site. Nuclear haplotypes of *A. hasseltii* in Mt. Howeishan and Lake Chunglingchih were identical except for the two minors (Hb1 and Hb2). However, only one major haplotype (Ha) was found in *A. hasseltii* and *A. subreflexipinna* of Mt. Kentuerhshan. The Hb and derivatively minor haplotypes were found neither in *A. hasseltii* nor *A. subreflexipinna* of Mt. Kentuerhshan.

TABLE 3. The variable nucleotide sites (indel & base substitution) of chloroplast *trnS-rps4* intergenic spacer sequences. Columns shaded are sites identical to the hybrid sequences.

Species	Variable sites																					
	0	0	0	0	0	1	1	1	1	2	2	2	2	2	2	2	2	2	3	3	3	3
	3	7	7	8	8	4	4	4	9	0	1	1	1	3	4	5	5	6	2	4	4	7
	9	2	6	2	4	0	1	2	3	8	2	4	7	0	1	2	8	8	7	7	8	3
A. diffracta	A	T	G	G	G	-	-	T	A	T	A	G	C	C	C	T	A	C	C	G	A	T
A. subreflexipinna	G	G	A	G	A	-	-	C	G	C	C	G	C	T	T	C	C	C	T	G	G	C
A. hasseltii	G	G	A	G	A	-	-	C	G	C	C	G	C	T	T	C	C	C	T	G	G	C
D. polita	A	G	G	T	G	T	T	T	G	T	C	C	T	C	C	C	C	T	T	A	G	T

The level of divergence among the three populations could not be revealed by the organellar fragments because only one haplotype was detected in each species (Tables 2–4). For nuclear *pgiC* intron 14–15 sequences, however, DnaSP analysis revealed high levels of genetic differentiation among three populations of *A. hasseltii* and *A. subreflexipinna* (γ_{ST} = 0.44377 and γ_{ST} = 0.26399; Table 7). Additionally, higher levels of genetic differentiation were also detected between northern and southeastern populations (A–B and A–C; Table 7) of these two species while little differentiation was found between those northern two (B–C; Table 7). For this same fragment, on the other hand, *A. diffracta* had only one haplotype and indicated no pattern of population structure.

DISCUSSION

Hybridization and parentage.—Similar to the traditional circumscription of species, hybrid species and hybrid parentage are usually postulated initially based on morphological characters and degree of spore/pollen abortion (Barrington, 1989, 1990). *Acrorumohra subreflexipinna* is suspected as a natural hybrid between *A. hasseltii* and *A. diffracta* (Moore, 2000) because *A. subreflexipinna* has abortive spores and intermediate morphology between *A. hasseltii* and *A. diffracta*, and occurs sympatrically with these two species. In addition, *A. subreflexipinna*'s spores show no germination, but those of *A. hasseltii* and *A. diffracta* germinate at a rate of more than 80% (unpublished data). Therefore, *A. subreflexipinna* appears to be a sterile F1 hybrid.

TABLE 4. The variable nucleotide sites (indel & base substitution) of mitochondrion *nad5* intron 2 sequences. Columns shaded are sites identical to the hybrid sequences.

Species	Variable sites														
	2	2	2	2	2	3	3	3	3	4	7	7	7	7	7
	1	1	1	1	1	7	7	8	8	1	0	0	0	0	1
	3	4	5	6	7	8	9	0	1	1	6	7	8	9	0
A. diffracta	T	G	G	T	T	T	-	T	T	T	C	C	G	C	T
A. subreflexipinna	-	-	-	-	-	C	A	C	C	T	-	-	-	-	-
A. hasseltii	-	-	-	-	-	C	A	C	C	T	-	-	-	-	-
D. polita	T	G	G	T	T	T	-	T	T	-	-	-	-	-	-

Acrorumohra subreflexipinna with its perennial habit, however, could occupy an original habitat for a long time despite of all spores being sterile. Repeated hybridization where the putative parents sympatrically exist might also replenish the stock of this hybrid.

Organelle genomes are generally maternally inherited in monilophytes (Gastony and Yatskievich, 1992; Vogel *et al.*, 1998). The assumption that chloroplast and mitochondria are maternally inherited is adopted through this study. All organellar sequence data indicated that *A. hasseltii* was the maternal parent of *A. subreflexipinna*. Nuclear *pgiC* sequences indicated that *A. diffracta* was the other genome donor of this hybrid.

In addition to the three taxa of *Acrorumohra* discussed here, another species, *A. yoroii* (Seriz.) Shieh, was reported in the second edition of Flora of Taiwan (Shieh *et al.*, 1994). In Taiwan, it grows in high montane regions and never sympatrically with other three taxa of *Acrorumohra*. Samples of that species were also collected from Taiwan and sequenced. It has organellar and nuclear sequences different from those of *A. subreflexipinna*, and phylogenetic analysis indicates a distant relationship between them (data not shown).

There are three other species of this narrowly defined genus. *Acrorumohra dissecta* Ching ex Hsieh is distributed in a few locations of southwestern China, and *A. obtusissima* (Mett. ex Kuhn) Ching and *A. undulata* (Bedd.) Ching are distributed throughout Sri Lanka. Though we cannot reject the hypothesis, the possibility of these species contributing to the formation of this hybrid is extremely low because of their restricted habitats and disjunct distribution from this hybrid.

TABLE 5. The haplotype and variable nucleotide sites (indel & base substitution) of nuclear *pgiC* intron 14–15 sequences. Columns shaded are sites different from those of other populations.

				Variable sites																									
		Haplotype code[b]	Frequency	0	0	0	1	1	1	2	2	2	2	2	3	3	3	3	4	4	4	4	5	5	5	5	6	6	6	6
Site[a]	Species			4	6	9	2	4	6	2	4	5	5	6	0	4	5	8	5	5	6	7	2	2	3	7	0	1	6	7
				8	3	1	2	4	9	6	7	3	4	6	8	4	0	1	0	1	5	8	2	3	4	3	9	0	4	0
	A. diffracta	D	10	C	G	A	G	C	C	T	A	T	G	C	T	A	G	A	C	A	C	T	-	-	A	G	C	A	T	T
		D	5	C	G	A	G	C	C	T	A	T	G	C	T	A	G	A	C	A	C	T	-	-	A	G	C	A	T	T
	A. subreflexipinna	Ha	1	T	G	T	C	T	T	C	G	C	T	C	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
A		Ha1	4	T	G	T	C	T	T	C	G	C	T	[T]	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
	A. hasseltii	Ha	6	T	G	T	C	T	T	C	G	C	T	C	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
		Ha1	4	T	G	T	C	T	T	C	G	C	T	[T]	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
	A. diffracta	D	10	C	G	A	G	C	C	T	A	T	G	C	T	A	G	A	C	A	C	T	-	-	A	G	C	A	T	T
		D	4	C	G	A	G	C	C	T	A	T	G	C	T	A	G	A	C	A	C	T	-	-	A	G	C	A	T	T
	A. subreflexipinna	Ha	2	T	G	T	C	T	T	C	G	C	T	C	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
B		Hb	2	T	[-]	T	C	[-]	T	C	G	C	T	C	C	A	A	[A C A G]			C	C	T	G	A	[-]	G	C	[T]
		Ha	2	T	G	T	C	T	T	C	G	C	T	C	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
	A. hasseltii	Hb	5	T	[-]	T	C	[-]	T	C	G	C	T	C	C	A	A	[A C A G]			C	C	T	G	A	[-]	G	C	[T]
		Hb1	3	T	[-]	T	C	[-]	T	C	G	C	T	C	C	[-]	A	A	[C A G]		C	C	T	G	A	[-]	G	C	[T]
	A. diffracta	D	10	C	G	A	G	C	C	T	A	T	G	C	T	A	G	A	C	A	C	T	-	-	A	G	C	A	T	T
		D	11	C	G	A	G	C	C	T	A	T	G	C	T	A	G	A	C	A	C	T	-	-	A	G	C	A	T	T
	A. subreflexipinna	Ha	5	T	G	T	C	T	T	C	G	C	T	C	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
C		Hb	6	T	[-]	T	C	[-]	T	C	G	C	T	C	C	A	A	[A C A G]			C	C	T	G	A	[-]	G	C	[T]
		Ha	4	T	G	T	C	T	T	C	G	C	T	C	C	A	A	G	-	-	C	C	C	T	G	A	T	G	C	G
	A. hasseltii	Hb	5	T	[-]	T	C	[-]	T	C	G	C	T	C	C	A	A	[A C A G]			C	C	T	G	A	[-]	G	C	[T]
		Hb2	1	T	[G]	T	C	[-]	T	C	G	C	T	C	C	A	A	[A C A G]			C	C	T	G	A	[-]	G	C	[T]

[a] A: Mt. Kentuerhshan, B: Lake Chunglingchih, C: Mt. Howeishan.

[b] First letter designated to different haplotypes originated from different species; D: *A. diffracta*, H: *A. hasseltii*. Lowercase letter and number designated to different minor haplotypes of *A. hasseltii*.

TABLE 6. Number of haplotypes, estimates of haplotype diversity (h) and nucleotide diversity (π) of *A. hasseltii* and *A. subreflexipinna*. The *A. diffracta* haplotype 'D' cloned from *A. subreflexipinna* were excluded prior to this analysis.

Populations	Species	No. of individuals	No. of haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)
Total (A+B+C)	*A. hasseltii*	30	5	0.724	0.00485
	A. subreflexipinna	20	3	0.674	0.00466
Mt. Kentuerhshan (A)	*A. hasseltii*	10	2	0.533	0.00074
	A. subreflexipinna	5	2	0.400	0.00055
Lake Chunglingchih (B)	*A. hasseltii*	10	3	0.689	0.00360
	A. subreflexipinna	4	2	0.667	0.00553
Mt. Howeishan (C)	*A. hasseltii*	10	3	0.644	0.00446
	A. subreflexipinna	11	2	0.545	0.00453

Although the phylogenetic analysis based on chloroplast *trnS-rps4* IGS sequence show a sister-group relationship between *D. polita* and *A. hasseltii* (Li and Lu, 2006; our unpublished data), this study reveals that *A. subreflexipinna* has sequences different from those of *D. polita* in both organellar (Tables 2–4) and nuclear (data not shown) genomes. Therefore, *Dryopteris polita* did not contribute to the formation of this hybrid. These molecular data plus morphological and ecological information explicitly suggest that *A. subreflexipinna* arose through hybridization of *A. hasseltii* and *A. diffracta*, and that the former was its putative maternal parent while the latter was its paternal source.

Acrorumohra hasseltii is distributed in tropical Asia, including Java, Borneo, Thailand, Nepal, East Himalayas, Vietnam, Hainan, Taiwan and southern Japan (Fig. 1). The range of *A. diffracta* overlaps with *A. hasseltii* in the northern portion of the range of *A. hasseltii*, i.e., East Himalayas, northern Thailand, Vietnam, Hainan and Taiwan (Fig. 1). Except for southwestern China, the geographic range of *A. diffracta* almost completely overlaps with that of *A. hasseltii*. However, *A. subreflexipinna* has only been reported from Taiwan. It is suspected that *A. subreflexipinna* might be established at some sites across this widely overlapping range of these two putative parents but misidentified as *A. diffracta* because of their similar zigzag rachis. Careful recognition is needed to identify this hybrid in future field investigation where the range of these two species overlaps.

Gender bias in hybridization events has been demonstrated many times in plants (Emms *et al.*, 1996; Vogel *et al.*, 1998; Weiblen and Brehm, 1996; Xiang *et al.*, 2000). For reasons not entirely clear, the hybridization of *A. hasseltii* and *A. diffracta* in our study was absolutely biased, i.e., *A. hasseltii* always was the supplier of egg while *A. diffracta* was that of sperm. This phenomenon has also been found in other hybrid species (e.g., Arnold and Bennett, 1993; Peng and Chiang, 2000; Smith and Sytsma, 1990; Wendel *et al.*, 1991). In ferns, mating systems usually correlate with ploidy levels and could be a decisive factor in the nuclear-organellar combination pattern of parental genotypes in hybridization. In fact, *A. diffracta* was reported as a tetraploid (Tsai and Shieh, 1975)

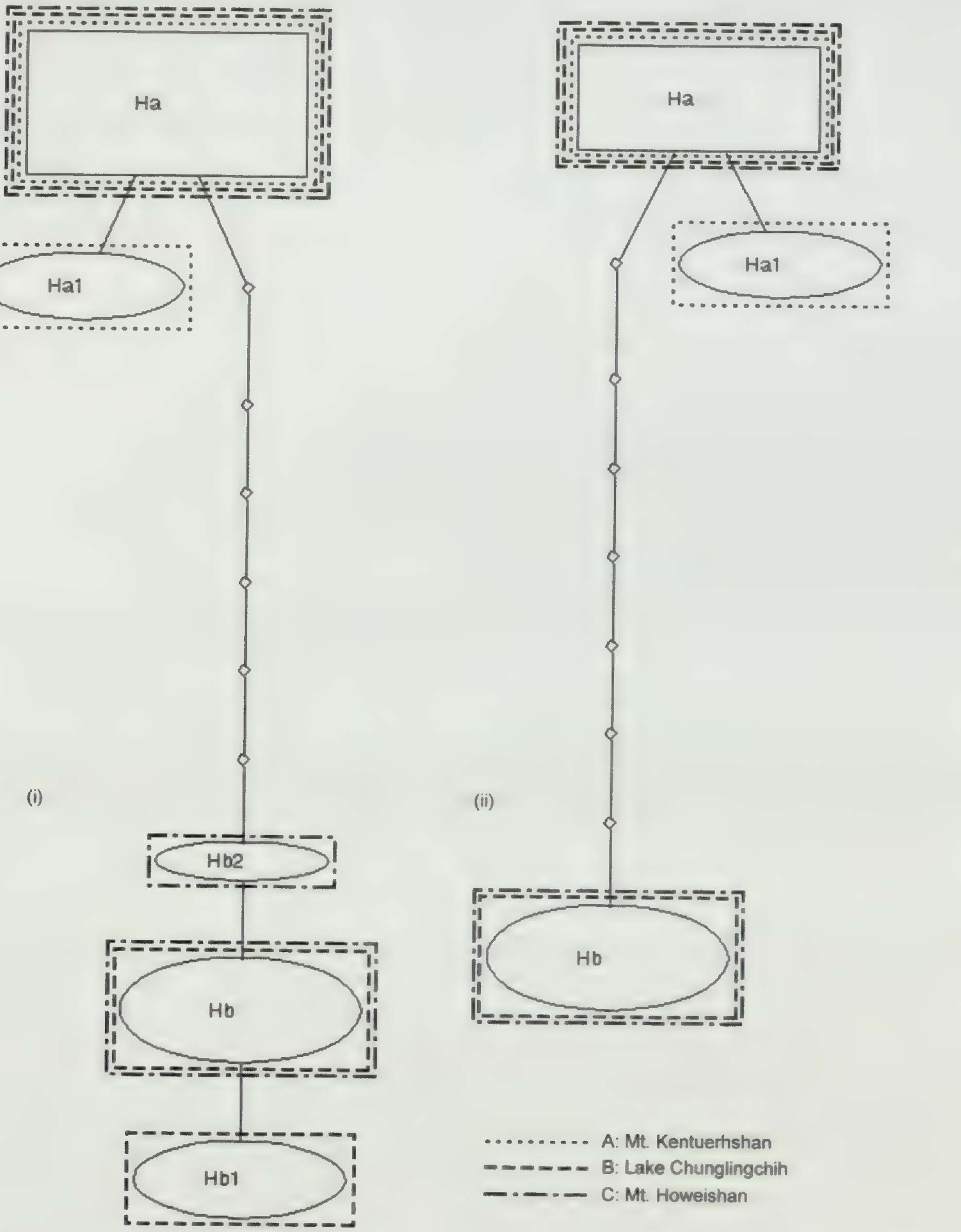

FIG. 2. *pgiC* intron 14–15 network for *Acrorumohra hasseltii* (i) and *A. subreflexipinna* (ii). Letters and number in the boxes or circles are haplotype codes. Dashed boxes indicate site(s) in which the haplotype is detected. The little rhombuses indicate the hypothetical haplotypes. Each line between haplotypes represents a mutational step.

and *A. hasseltii* a diploid (Iwatsuki, 1995), and our observation showed that *A. hasseltii* has 64 spores per sporangium but *A. diffracta* has 32 spores (unpublished data). These agree with the general rule that a sexual species usually has 64 spores per sporangium whereas there are typically 32 spores in an apogamous species. According to the Dopp-Manton Scheme and Braithwaite modes of reproduction (Raghavan, 1989), *Acrorumohra diffracta*

TABLE 7. Genetic differentiation between pairwise and among all populations of *A. hasseltii* and *A. subreflexipinna*.

Species	Genetic differentiation among all populations (γ_{ST})	Genetic differentiation between pairwise populations (γ_{ST})[a]		
		A–B	A–C	B–C
A. hasseltii	0.44377	0.60815	0.40303	0.06301
A. subreflexipinna	0.26399	0.40000	0.31460	0.00162

[a]A: Mt. Kentuerhshan, B: Lake Chunglingchih, C: Mt. Howeishan.

might be an obligate apogamous species with functional antheridia. These observations and molecular evidence lead to a convincing hypothesis for unidirectional hybridization that sexual *A. hasseltii* could adopt sperm from the apogamous tetraploid, *A. diffracta*. Additionally, a lack of heterozygosity of the *A. hasseltii* genotypes in this hybrid further supports that *A. hasseltii* was the haploid female gamete donor in these hybridization events. In this case, because fertile sperm were liberated from an apogamous, tetraploid gametophyte, *A. subreflexipinna* should be a pentaploid. C-value results based on flow cytometry show that *A. subreflexipinna* has the highest ploidy level among these taxa (unpublished data), which confirms this hypothesis. Further observations on chromosome counts and the mating system of *A. subreflexipinna* and both parents in the future would provide more direct evidence.

Multiple independent origins.—Although *A. subreflexipinna* has a larger body size than both putative parental species, it produces no fertile spores, and its small population size and rare occurrence strongly suggests it is a product of occasional hybridization event(s). Both cpDNA and mtDNA fragments of populations of *A. hasseltii* and *A. subreflexipinna* were identical in sequence. Single direction hybridization and allopolyploidization may produce cytoplasmically uniform hybrids or allopolyploids despite distinct origins (Soltis *et al.*, 1992). The variation in cpDNA and mtDNA from the taxa of this study was uninformative regarding multiple origins of *A. subreflexipinna*. Nuclear *pgiC* sequences, however, revealed different haplotypes and genetic differentiation among populations of *A. hasseltii* and *A. subreflexipinna*. Southern and northern populations of *A. hasseltii* had distinct and unique genotypes, and the genetic uniqueness was transmitted to their hybrid offspring (Table 5; Fig. 2). In both *A. hasseltii* and *A. subreflexipinna*, there were nine variable sites that had different bases in southern and northern populations (Table 5). Haplotype Ha1 was found in *A. hasseltii* of Mt. Kentuerhshan and was present in *A. subreflexipinna* in the same location. On the other hand, major haplotype Hb was only found in the northern populations of both species but not in the southern ones (Fig. 2). These are direct indicators of multiple independent origins of *A. subreflexipinna*.

There was no variation of organellar DNA fragments in populations of *A. hasseltii* and *A. subreflexipinna*, but high nucleotide diversity was found in the nuclear *pgiC* intron 14–15 of the same species. The fact that, usually,

evolutionary rates of nuclear DNA are faster than those of chloroplast and mitochondria in plants (Graur and Li, 2000) may provide a reasonable interpretation of this significant difference. This also indicates that nuclear DNA markers may be a better choice when analyzing population variation due to relatively short evolutionary time. In this case, geographical barriers might effectively hinder gene flow, causing geographical subdivision in nuclear DNA, but the time of isolation might not have been long enough to accumulate new mutations in organellar DNA.

Several studies indicate that multiple origins of hybrid and polyploid species are common, if not the rule, in plants (see Soltis *et al.*, 1992 and references therein). Genetic variation of the parental species could be incorporated into and preserved in hybrids and their derivative taxa by these processes (Arft and Ranker, 1998; Paun *et al.*, 2007; Peng and Chiang, 2000). This phenomenon was also found in this study. Unlike a small population usually having fixed alleles for most loci, populations of *A. subreflexipinna*, even when only four individuals were found in a population, have high haplotype diversity. Moreover, the results agree with our anticipation that in each collecting site there were fewer haplotypes and lower haplotype diversity of *A. subreflexipinna* than those of *A. hasseltii*. Because *A. subreflexipinna* is a sterile hybrid, any genetic variation should come from its parents. It is the recurrent hybridization of this hybrid that might maintain its significant genetic variability and provide operative materials for future evolution, i.e., polyploidization and subsequent fertilization.

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Molecular Cloning and Sequence Analysis of Cyanovirin-N Homology Gene in *Ceratopteris thalictroides*

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ABSTRACT.—A new full-length genomic DNA, encoding a member of the cyanovirin-N (CV-N) homologous protein family, has been cloned from the fern species *Ceratopteris thalictroides* by chromosome walking. It is 1993 bp long, contains a 723 bp open reading frame (ORF) that encodes a deduced protein (named CtCVNH) with 150 amino acid residues. CtCVNH has a predicted isoelectric point (PI) of 4.47 and a calculated molecular mass 15.9556 kDa. It possesses the conserved anti-HIV (human immunodeficiency virus) CV-N domain, which is the same as the cyanovirin-N homology (CVNH) members that were isolated from filamentous ascomycetes and *C. richardii*. Modeling of the tertiary structure indicated that CtCVNH is an elongated, largely β-sheet protein that displays internal two-fold pseudosymmetry. Comparative structure analysis of the predicted CtCVNH with native CV-N revealed that the major evolutionary changes occurring during the evolution of plant CVNHs were: 1) a length increase at N- and C-terminal regions; and 2) a loop to helix transition at the helical-turn regions. Phylogenetic analysis showed that CtCVNH was grouped together with the two CVNHs from *C. richardii*.

KEY WORDS.—*Ceratopteris thalictroides*, chromosome walking, single oligonucleotide nested PCR, inverse PCR, thermal asymmetric interlaced PCR, CVNH, bioinformatic analysis

Cyanovirin-N (CV-N) is an 11 kDa anti-HIV (human immunodeficiency virus) protein originally isolated from the extract of the cyanobacterium *Nostoc ellipsosporum* (Des.) Rabenh. (Boyd *et al.*, 1997). It consists of a single chain with 101 amino acids, exhibits significant internal sequence duplication between residues 1–50 and 51–101, and contains two intramolecular disulfide bonds (Gustafson *et al.*, 1997). CV-N is largely comprised of β-sheets with a two-fold pseudosymmetry (Bewley *et al.*, 1998). Its antiviral activity depends on the high-affinity binding to the HIV surface envelope glycoprotein, gp120 (Boyd *et al.*, 1997; Mori *et al.*, 1997). CV-N can specifically interact with high mannose groups (Bolmstedt *et al.*, 2001; Botos *et al.*, 2002), thereby blocking the interaction between gp120 and the receptor CD4 on target cells (O'Keefe *et*

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al., 1997). Besides HIV strains (Boyd *et al.*, 1997), CV-N is also able to inactivate simian immunodeficiency virus (SIV), Ebola virus (EBO), herpes simplex virus-1 (HSV-1), and hepatitis C virus as well (Barrientos *et al.*, 2003; O'Keefe *et al.*, 2003; Barrientos and Gronenborn, 2005; Helle *et al.*, 2006). The potent inactivation of HIV plus unique biophysical properties make CV-N a candidate for a topical anti-HIV microbicide. The CV-N preclinical development is underway (Colleluoria *et al.*, 2005).

Recently, a family of CVNH (cyanovirin-N homology) has been identified. All CVNH proteins share a common fold that matches the one previously thought to be unique in CV-N (Percudani *et al.*, 2005). Current research on CVNHs is mainly focused on structural information, antiviral activity, carbohydrate-binding specificities or structure-function relationships (Percudani *et al.*, 2005; Koharudin *et al.*, 2008). For example, solution structures of three CVNHs from *Tuber borchii* Vittad., *Ceratopteris richardii* Brongn., and *Neurospora crassa* Shear et Dodge have been determined (Koharudin *et al.*, 2008) and may be helpful in elucidating the roles that these proteins play in the organs and during evolution.

CVNHs show a patchy organism distribution regarding the anti-HIV domain. They are present in organisms as diverse as cyanobacteria, filamentous ascomycetes and seedless plants (Percudani *et al.*, 2005). However, among plants, CVNHs have only been identified in the fern *C. richardii* until now. To provide useful information for understanding the evolution of CVNHs and developing antiviral polypeptides, here we report the cloning and sequence analysis of the full-length CVNH genomic DNA in *Ceratopteris thalictroides* (L.) Brongn. together with an analysis of CVNHs phylogeny and modeling of the protein tertiary structure.

Materials and Methods

Plant materials.—*Ceratopteris thalictroides* was collected from Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan, Hubei, China. Young and healthy leaves were sampled, immediately frozen in liquid N_2, and stored at −70°C until used.

Genomic DNA extraction.—Total genomic DNA was extracted from fresh leaves following the modified CTAB protocols (Su *et al.*, 1998). DNA concentration and purity were determined by measuring UV absorption using a Pharmacia 2000 UV/Visible spectrophotometer. DNA intactness was checked by 1.0% agarose gel electrophoresis.

Molecular cloning of the full-length genomic DNA.—Based on the *C. richardii* EST sequence (Accession No. BQ087187), specific primers were designed to amplify the internal region of *CVNH* in *C. thalictroides*. The forward primer CVNH-F was 5′-GTGGGCGTCTAGCGATTTCCTTT-3′, and the reverse primer CVNH-R was 5′-ATCATCCGCTGCTTGCTTCTTCG-3′. The reaction mixture (20 μL) contained 50 ng template DNA, 40 pmol each primer, 1 pmol each dNTP, 1.0 U *Taq* DNA polymerase and 1 × *Taq* polymerase buffer. PCR was performed using the following protocol: the template was

TABLE 1. The primers used in chromosome walking.

Primer name	Primer sequence
5′IPCR	5′-GGTGATATTGCCCGTCGGTGCCTTT-3′
5′SON-1	5′-ATCACTGTTGAGGCAATCTGCGGCT-3′
5′SON-2	5′-GCTGCGATCAAGACGATGAGAAAAC-3′
5′SON-3	5′-CCATCGCTTCTAGGAGTAAACAGAC-3′
3′TAIL-1	5′-GTGCAAAGGCACCGACGGGCAATAT-3′
3′TAIL-2	5′-GGGGTGTTGGATTTCTGTGGCTATG-3′
3′TAIL-3	5′-AAGCGAAGAAGCAAGCAGCGGATGA-3′

denatured at 94°C for 5 min followed by 36 cycles of amplification (94°C for 50 s, 61°C for 50 s, 72°C for 90 s) and a final extension of 10 min at 72°C.

Based on the sequence obtained from the internal DNA region, two sets of nested primers for 5′ single oligonucleotide nested PCR (SON-PCR) (Antal *et al.*, 2004) and 3′ inverse PCR (IPCR) (Triglia *et al.*, 1988) combined thermal asymmetric interlaced PCR (TAIL-PCR) (Liu and Whittier, 1995) were designed to amplify the 5′ and 3′ flanking sequences. These primers included 5′IPCR, 5′SON-1, 5′SON-2, 5′SON-3, 3′TAIL-1, 3′TAIL-2, and 3′TAIL-3 (Table 1, Fig. 1). They were of high annealing temperatures and synthesized by Invitrogen (Shanghai).

The 5′ flanking sequence was amplified by SON-PCR. The primary PCR was carried out in a 20 μl volume containing 50 ng genomic DNA, 50 pmol single primer (5′SON-1), 50 mol/L each dNTP, 2.0 U *Taq* DNA polymerase and 1 × *Taq* polymerase buffer. For the secondary PCR, two single primers (5′SON-2 and 5′SON-3) were separately used. The reaction solution was the same as that of primary PCR except that 1 μl of a 1:50 dilution of the primary PCR products was used as the template.

The 3′ flanking sequence was obtained using IPCR combined TAIL-PCR. *Ceratopteris thalictroides* genomic DNA was digested with Pac I (NEB, BSA 5 U μg^{-1} of DNA) at 37°C for 3 h, and then heated at 65°C for 20 min. The digested DNA was self-ligated overnight at 15°C with a concentration of 0.3–0.5 μg/ml in the presence of 3 U/ml T4 DNA ligase (Promega). PCR was carried out in a 20 μl volume with 1 μl ligated product, 1 pmol each dNTP, 40 pmol each primer (5′IPCR and 3′TAIL-1), 1.0 U *Taq* DNA polymerase and 1 × *Taq* polymerase buffer. The primary PCR of TAIL-PCR was performed using primer

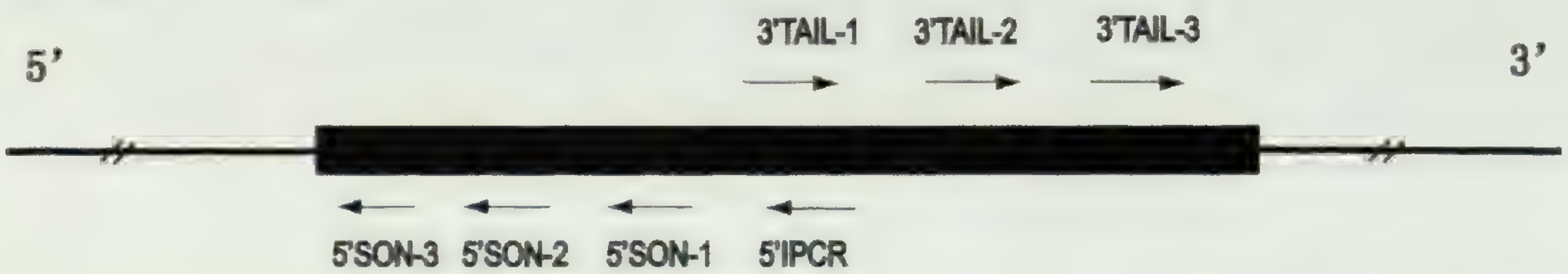

FIG. 1. Schematic view of position and orientation of nested primers used in this study and of their relative positions to the amplified sequence of the specific PCR. The rectangle frame indicates the sequence obtained by specific PCR, whereas the line represents regions determined by further chromosome walking.

TABLE 2. Cycling conditions used for SON-PCR, IPCR, and TAIL-PCR.

Name	Reaction	Cycle no.	Thermal condition
5′SON-PCR	Primary	1	94 °C (5 min)
		5	94 °C (30 s), 65 °C (1 min), 72 °C (2.5 min)
		1	94°C(30 s), 29°C(3 min), ramping to 72°C over 3 min, 72°C(2.5 min)
		60	94°C(30 s), 65°C(1 min), 72°C(2.5 min)
		1	72°C(7 min)
	Secondary	1	94°C(5 min)
		30	94°C(30 s), 65°C(1 min), 72°C(2.5 min)
		1	72°C(7 min)
3′IPCR	IPCR	1	94°C(10 min)
		33	94°C(1 min), 65°C(1 min), 72°C(2 min)
		1	72°C(10 min)
3′TAIL-PCR	Primary	1	93°C(1 min), 95°C(1 min)
		5	94°C(30 s), 65°C(1 min), 72°C(2.5 min)
		1	94°C(30 s), 25°C(3 min), ramping to 72°C over 3 min, 72°C(2.5 min)
		15	94°C(30 s), 65°C(1 min), 72°C(2.5 min)
			94°C(30 s), 65°C(1 min), 72°C(2.5 min)
			94°C(30 s), 44°C(1 min), 72°C(2.5 min)
		1	72°C(5 min)
	Secondary	1	94°C(1 min)
		15	94°C(30 s), 65°C(1 min), 72°C(2.5 min)
			94°C(30 s), 65°C(1 min), 72°C(2.5 min)
			94°C(30 s), 44°C(1 min), 72°C(2.5 min)
		1	72°C(5 min)

3′TAIL-1 as the gene-specific primer and primer AD (5′-TC(G/C)TICGNA-CIT(A/T)GGA-3′) (Liu and Whittier, 1995) as the arbitrary degenerate primer in a total 20 µl volume that contained 1 µl of a 1:50 dilution of the IPCR products, 2 pmol each dNTP, 40 pmol primer 3′TAIL-1, 500 pmol primer AD, 2.0 U *Taq* DNA polymerase and 1 × *Taq* polymerase buffer. For the secondary PCR, two gene-specific primers (3′TAIL-2 and 3′TAIL-3) were separately used with the same arbitrary primer as used in the primary one. The reaction solution was the same as that used for the primary PCR except that 1 µl of a 1:50 dilution of the primary PCR products was used as the template. Thermocycling profiles used for SON-PCR, IPCR, and TAIL-PCR are listed in Table 2.

Recovery of PCR products.—PCR products were purified by running them through a 1.0% low melting agarose gel. The desired DNA band was cut out and recovered using the DNA rapid purification kit (Omega).

DNA cloning and sequencing.—A purified PCR product was ligated into a pMD 19-T (TaKaRa) vector and then used to transform competent *Escherichia coli* cells DH-5α. A positive clone was identified by blue/white selection and ascertained by PCR. Purified plasmid DNA was sequenced in both directions by standard methods on an ABI 3730 automated sequencer at Invitrogen (Shanghai). Primers M13F and M13R located on pMD19-T vector were utilized for sequence determination.

In silico *analysis and molecular modeling.*—ORF finder was used to predict coding sequence, and promoter analysis was performed online (http://www.fruitfly.org/cgi-bin/seq_tools/promoter.pl). Sequence analysis was conducted using the BLAST program (Altschul *et al.*, 1997) and other programs available at the ExPASy server (Gasteiger *et al.*, 2003). Multiple sequence alignment was carried out using the ClustalX software (Thompson *et al.*, 1997). Figures of multiple sequence alignment adorned with secondary structure elements were generated with ESPript (Gouet *et al.*, 1999). Primary structure analysis of the deduced CtCVNH (CVNH protein from *C. thalictroides*) was conducted with ProtParam (Gasteiger *et al.*, 2005) by using the ExPASy server online (http://www.expasy.ch/tools/protparam.html). Secondary structure was predicted with SOPMA program (Geourjon and Deleage, 1995) online (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html). Phylogenetic analysis was carried out using programs from the PHYLIP package; genetic distances were estimated with PROTDIST using the Jones-Taylor-Thornton model of amino acid substitutions. Neighbor-joining trees (Saitou and Nei, 1987) were constructed using the NEIGHBOR program; 1000 random replications were utilized for bootstrap analysis, which was performed with the SEQBOOT and CONSENSE programs. Phylogenetic trees were rendered with the TREEVIEW program (Page, 1996). The three-dimensional (3D) structural models of CtCVNH were built by the homology-based method using the SWISS-MODEL program (Guex and Peitsch, 1997; Schwede *et al.*, 2003; Arnold *et al.*, 2006). The template used for modeling was *C. richardii* CVNH (PDB code 2jzjA) (Koharudin *et al.*, 2008). Models were displayed with the PyMol program (Delano, 2002).

Results and Discussion

Molecular cloning of the full-length genomic DNA.—Using a pair of specific primers (CVNH-F and CVNH-R), a single fragment of 775 bp was amplified from the *C. thalictroides* DNA [Fig. 2(a)]. Compared with the *C. richardii* cDNA sequence (Accession No. BQ087187), the sequence from *C. thalictroides* has two additional fragments that do not exist in *C. richardii* cDNA and the remaining parts of the sequence are identical to the *C. richardii* cDNA (Fig. 3). The *CtCVNH* intron–exon boundaries were thus deduced; it is composed of three exons and two introns. Based on the amplified sequence of the specific PCR, two sets of nested primers were further designed to obtain the 5′ and 3′ flanking sequences, respectively. A clear single band ≈ 800 bp of the 5′ flanking sequence was generated in the secondary reaction [Fig. 2(b)] using SON-PCR, while a ≈ 750 bp 3′flanking sequence was amplified through IPCR combined TAIL-PCR [Fig. 2(c)].

Sequence analysis of the CtCVNH *gene.*—The cloned full-length *CtCVNH* gene is 1993 bp in length, including a 818 and 452 bp 5′ and 3′ untranslated region (UTR) respectively, and a 723 bp coding region. The 5′UTR has a TATA box in the predicted promoter elements. The ATG start codon, which is numbered +1 to +3, is flanked by G in both positions -3 (3 nucleotides before

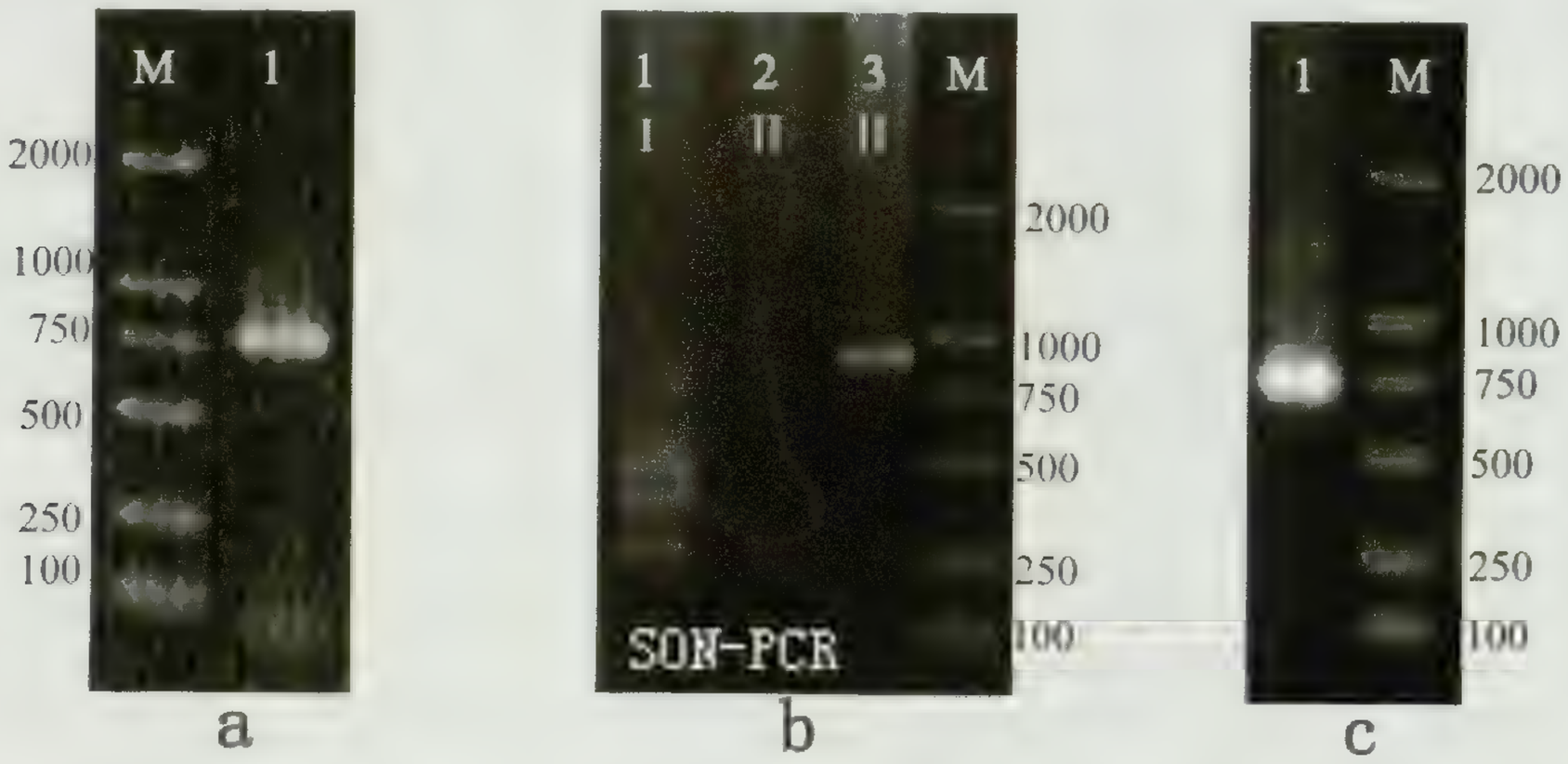

FIG. 2. Agarose gel electrophoresis of the specific PCR(a), SON-PCR(b), and IPCR+TAIL-PCR(c) products. M is molecular weight marker (DL2000). a 1: about 750 bp fragment generated by specific PCR with primer CVNH-F and CVNH-R. b 1: smear bands produced by the first reaction of SON-PCR with primer 5′SON-1. 2: no clear band produced by the secondary reaction of SON-PCR with primer 5′SON-2. 3: a single band obtained by the secondary reaction of SON-PCR with primer 5′SON-3. c 1: the amplified DNA fragment of secondary reaction of TAIL-PCR with primer 3′TAIL-2 and AD.

the ATG codon) and +4 (1 nucleotide after the ATG codon), indicating that it is located in a sequence context for strong translational initiation (Kozak, 1999). The 3′UTR has a polyadenylation signal (AATAAA) and six ATTT domains (Fig. 4). These ATTT domains may be important for mRNA destabilization (Shaw and Kamen, 1986). The *CtCVNH* gene encodes a deduced protein of 150 amino acid residues with a predicted isoelectric point (pI) of 4.47 and a calculated molecular mass of 15.9556 kDa. Regarding its amino acid composition, the most abundant is Ser (13.3% by frequency), followed by Gly (9.3%), Leu (9.3%), Ala (7.3%), Asn (7.3%), Asp (7.3%), and Val (6.7%). Acidic and basic amino acids constitute 10.0% and 5.3% of the protein, respectively. Moreover, 15.3% of the amino acids are charged, and the percentages of polar and hydrophobic amino acids are 64% and 25.3%, respectively (Table 3).

With regard to the predicted secondary structure, the CtCVNH protein consists of 16.00% alpha helices, 28.67% extended strands, 12.67% β turns, and 42.67% random coils. The extended strands and random coils constitute the interlaced domain of the main part of the secondary structure.

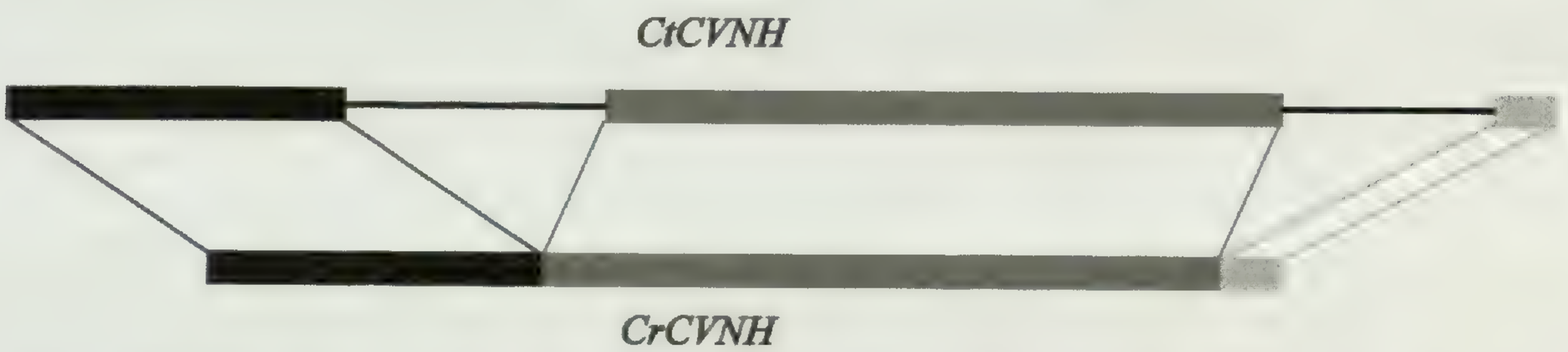

FIG. 3. Schematic view of the exon and intron positions deduced from *C. richardii* cDNA. The exon and intron are indicated by rectangle frame and line, respectively.

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                                                CCATCGCTTCTAGGAGTAAACAGACATACTATAACTGC   -781
ACTCTAACACATTTTCAACAATACAAGAAACATTTATACATACTTCACACGTGCACATCA   -721
TCACACATACACACATACTATGTGTGCATACATGCTCACATGCATAGTAGCATGCATATA   -661
CACATAGTAAATAATGCACATGCATACATATATACACATGCACATTATTTCGGTGGGCCT   -601
TCAAATGCTAGTTAAAAATTTATTGTTATTATCTTTTTAAATGGAGTTCCGGCAAGTGGT   -541
TACTCCCCCGAGCTTTAACTTTGAAAGAATAACTTAACACGTGAATGGACATAGTGTTCG   -481
AGGGACCCCTCGTATCCCACAAAGTAAGAGTTGGATAAAATTACAACTTGTTTCCATTGG   -421
TTTAGACTGGCCAAGGCCATTACACGCTCAAGCCAGAGATTGAACTTTGAACTTCCACCT   -361
GAAGAGAGCAAAGACTCAACCACTAGAACAACGAAATGTGAACTTAAAAATTTATTATTG   -301
GTTGTTTTGAAAAAAATTCTTTATTACAATCTTAATTTTCATTTGATTGCCCAATTTTTA   -241
GAAAGGCAGTCACGGAGACCAAACGCTAAGTCCACTGCTCGTTTGCAAAGGAGAACGTTT   -181
GTCTTTATCGAGTATTATATACAGAAACGCTAAGTTGCGCCACATGTCATGAAATAAGCG   -121
TCCGAGATCCAAAGACGTTAACTTTCCCTACTATATAAATGGGGAAGCTTGAGACCATCT    -61
GCCCAGTCCGTTATTCGTCTAGCGATTTCCTTTCGAAGTGTCTGTTTACTCCTAGAAGCG     -1
ATGGAGTACTTTACACCGCGATATCTTCTCCTTCAAGGTTTTCTCATCGTCTTGATCGCA     60
M  E  Y  F  T  P  R  Y  L  L  L  Q  G  F  L  I  V  L  I  A
GCATCGAATGCCAGCGCTCAAgtaagtctttctgatttccagtttcatatgttaaatctc    120
A  S  N  A  S  A  Q
tctctctctctctctctctctctctctctctcccaattctggtttgtgatgttgtatc     180
atcgttctcgtctgcaTGCGATTTCTCATACTCGTGCAAGGATGTGACTGTAACCGGCAA    240
                  C  D  F  S  Y  S  C  K  D  V  T  V  T  G  N
CTTCCTAGCCGCAGATTGCCTCAACAGTGATGGTGCATACGATCGGTCTTCTCTGAATAT    300
 F  L  A  A  D  C  L  N  S  D  G  A  Y  D  R  S  S  L  N  M
GAACGACATGATTGGTAATAGTAATGGAAGGCTTGTATTTCCCGGTACCTCCTTCCGTAA    360
 N  D  M  I  G  N  S  N  G  R  L  V  F  P  G  T  S  F  R  N
TTCATGCTTGAGTGTGGAGATCAACGACGGTCATACGCTCACAGCTTCGTGCAAAGGCAC    420
 S  C  L  S  V  E  I  N  D  G  H  T  L  T  A  S  C  K  G  T
CGACGGGCAATATCACCCTACAAGCCTTGATCTCAACTCTTGCGTTTATAATGCTGACGG    480
 D  G  Q  Y  H  P  T  S  L  D  L  N  S  C  V  Y  N  A  D  G
GGTGTTGGATTTCTGTGGCTATGGTGTCGGAAAATCAACAGCCTACGTCAAGTCCAGTAC    540
 V  L  D  F  C  G  Y  G  V  G  K  S  T  A  Y  V  K  S  S  T
CGTgtaatgtccctgcaacaagtactgccgtagttatattatatatcgttacttcacct     600
 V
ctcaggaaatgctttactttgcgctctacaacactagctattctacttatatatggcatt    660
tgctggacgttgtttatattaattttctttctcttcacAAGCGAAGAAGCAAGCAGCGGA    720
                                      S  E  E  A  S  S  G
TGATTTGAGCATGTGCTTCACTGTCTGCAAACTTCCTCTATTATGCTGTGACGTTGTCTA    780
*
TCGAAGCCACACATCGGATTATATATAACATAGCGTCTTTTCATTTGGGTGATATGCTCC    840
TCGCGTTCTCTTTCCCTGCTCTTTCTTTGTCTTTGTTATGCCGCCTCAAGTTCTGCATGT    900
ATGATTTTATTCCTACGTGCTGTATTGATATCAGTAGATGTGTCCTATATTCATTTCACC    960
CTTCATTAATTAAGTATGTCAATGTCAACAGAAGTTACTCTCTAATAAAATCCATACGAA   1020
ATCTCCTGCATTCAGAAACCCACAAATAGAGAATTTCTAACAATGTCAGTTTATTACGTC   1080
TATTTATTCATAACCCACATCTAACTAACGCGAGTTACACTATAGGAAATCTCTTTCTAC   1140
TATTTTGGATTGTTAGTTTATTACGTCTATTTATT                            1175
```

FIG. 4. Nucleotide and deduced protein sequences of *CtCVNH* gene. The predicted amino acid sequence is shown below its open reading frame. The predicted promoter sequence is shown in shaded box (transcription start site shown in larger font). The TATA box is boxed with solid lines. The polyadenylation signal is underlined and boldface. The ATTT regions of the 3′UTR are underlined. The introns are present in lowercase letters.

TABLE 3. Component analysis of amino acid sequence of CtCVNH.

Amino acid	Number	Frequency(%)
Hydrophobic amino acid	38	25.3
Charged amino acid	23	15.3
Polar amino acid	96	64.0
Acidic amino acid	15	10.0
Basic amino acid	8	5.3
Ala(A)	11	7.3
Gly(G)	14	9.3
Met(M)	3	2.0
Ser(S)	20	13.3
Cys(C)	7	4.7
His(H)	2	1.3
Asn(N)	11	7.3
Thr(T)	10	6.7
Asp(D)	11	7.3
Ile(I)	4	2.7
Pro(P)	3	2.0
Val(V)	10	6.7
Glu(E)	4	2.7
Lys(K)	4	2.7
Gln(Q)	3	2.0
Trp(W)	0	0.0
Phe(F)	7	4.7
Leu(L)	14	9.3
Tyr(Y)	8	5.3
Arg(R)	4	2.7

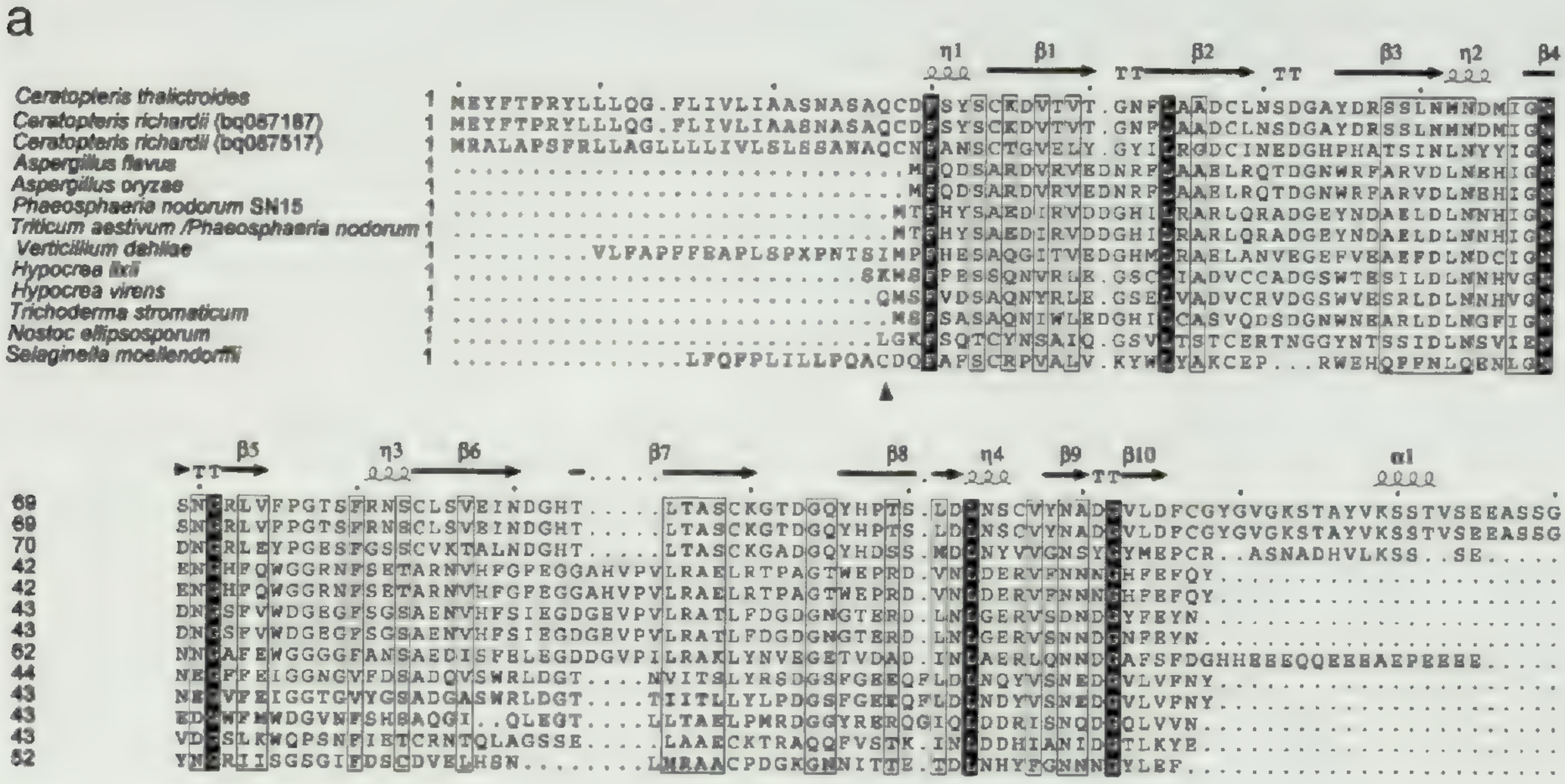
Amino acid sequence alignment and phylogenetic analysis.—Initial homology searches were conducted with the deduced CtCVNH amino acid sequence in the non-human, non-mouse EST database at the NCBI (National Center for Biotechnology Information, NIH, Bethesda) by using the tblastn program (Altschul *et al.*, 1997). A new member of CVNHs was uncovered from the plant *Selaginella moellendorffii* Hieron. by conducting these searches. The results (Table 4) showed that the CVNH members were present in fungi and plants (E value < 0.01). Above 70% of the members occurred in fungi. A comparison of the deduced CtCVNH against other CVNHs revealed that CtCVNH shares a high degree of similarity with the two CVNHs from *C. richardii* (99% and 53% identity, respectively), and a reduced level of similarity with the CVNHs from fungi (26–33%). Multiple sequence alignment indicated that the anti-HIV domain is conserved [Fig. 5(a)]. The most conservative sites were F4, L18, G27, L36, G41, N42, G45, F54, L69, G78, L87, N93, and G96 (the numbering is in line with the *N. ellipsosporum* CV-N). These residues are predominantly located in the hydrophobic core region, which are involved in hydrophobic interactions between the β-hairpin and the underlying triple-stranded β-sheet of each repeat (Percudani *et al.*, 2005). Also conserved are hydrophilic amino acids involved in the formation of the hydrogen-bonded bridges that connect

TABLE 4. Members of the CVNHs identified in nucleic acid databases.

Accession[a]	Source	Amino acid	Organism	Classification	Percent ID[b]	E[c]
BQ087187	mRNA	150	*Ceratopteris richardii*	Filicales	99	2e-81
BQ087517	mRNA	142	*Ceratopteris richardii*	Filicales	53	3e-24
FE488500	mRNA	106	*Selaginella moellendorffii*	Selaginellales	31	0.002
CO138997	mRNA	106	*Aspergillus flavus*	Eurotiomycetes	30	1e-05
EY428974	mRNA	106	*Aspergillus oryzae*	Eurotiomycetes	30	1e-05
EH395121	mRNA	106	*Phaeosphaeria nodorum* SN15	Dothideomycetes	33	2e-04
AJ917737	mRNA	101	*Trichoderma stromaticum*	Sordariomycetes	30	9e-05
DR044956	mRNA	106	*Triticum aestivum /Phaeosphaeria nodorum* mixed EST library	-	33	1e-04
BQ110230	mRNA	143	*Verticillium dahliae*	Sordariomycetes	26	0.002
AJ915102	mRNA	105	*Hypocrea lixii*	Sordariomycetes	33	0.003
FG382361	mRNA	104	*Hypocrea virens*	Sordariomycetes	33	0.004

[a]For protein sequences deduced from mRNA data, the accession number of a representative EST sequence is reported.
[b]Percent identity with respect to the deduced CtCVNH.
[c]Expected values for pairwise comparisons (blosum62 matrix) based on the size of the non-human, non-mouse EST database.

a



b

first half MEYFTPRYLLLQGFLIVLIAASNASAQCDFSYSCKDVTVT-GNFLAADCLNSDGA

second half ----------GTSFRNSCLSVEINDGHTLTASCKGTDGQ

.* ** .* :. *: *:*.* .:**

first half YDRSSLNMNDMIGNSNGRLVFP----------

second half YHPTSLDLNSCVYNADGVLDFCGYGVGKSTAYVKSSTVSEEASSG

*. :**::*. : *::* * *

FIG. 5(a). Multiple alignment of the CVNHs. Sequence conservation is visualized according to the ESPript chemical equivalence measure, with a similarity threshold set to emphasize strictly conserved positions. Invariant residues are boxed in black; physico-chemical equivalent residues are boxed in gray. The deduced secondary structure of CtCVNH is shown above the alignment. The predicted N-termini of the mature fern polypeptides are indicated by a black triangle. Fig. 5(b). Comparison of the amino acid sequences of domains 1–76 and 77–150 of CtCVNH. Sequence homology of the domains was maximized by insertion of gaps (-). Identical amino acids (·) and conserved amino acids (*) are indicated.

β-strands 1–9 and 4–6 (Bewley *et al.*, 1998). These suggest a critical structural role or their involvement in carbohydrate binding.

Sequence similarity was also examined between the first (residues 1–50, according to the numbering in the *N. ellipsosporum* CV-N) and the second half (residues 51–101) of the CVNHs. Like CV-N, all CVNHs comprise two tandem sequence repeats with identities ranging from 24.0% to 41.1% (data not shown). Several residues are completely conserved [Fig. 5(b)]. The apparent sequence similarity between the two repeats (with an average identity of 33.3%) can be ascribed to the structural constraints imposed by the symmetrically interconnected CVNH fold (Percudani *et al.*, 2005).

A neighbor-joining tree (Fig. 6) was constructed to analyze the phylogenetic relationships of CtCVNH with other CVNHs (Table 4). It shows that CtCVNH is closely related to the member from *C. richardii* (BQ087187), and CVNHs

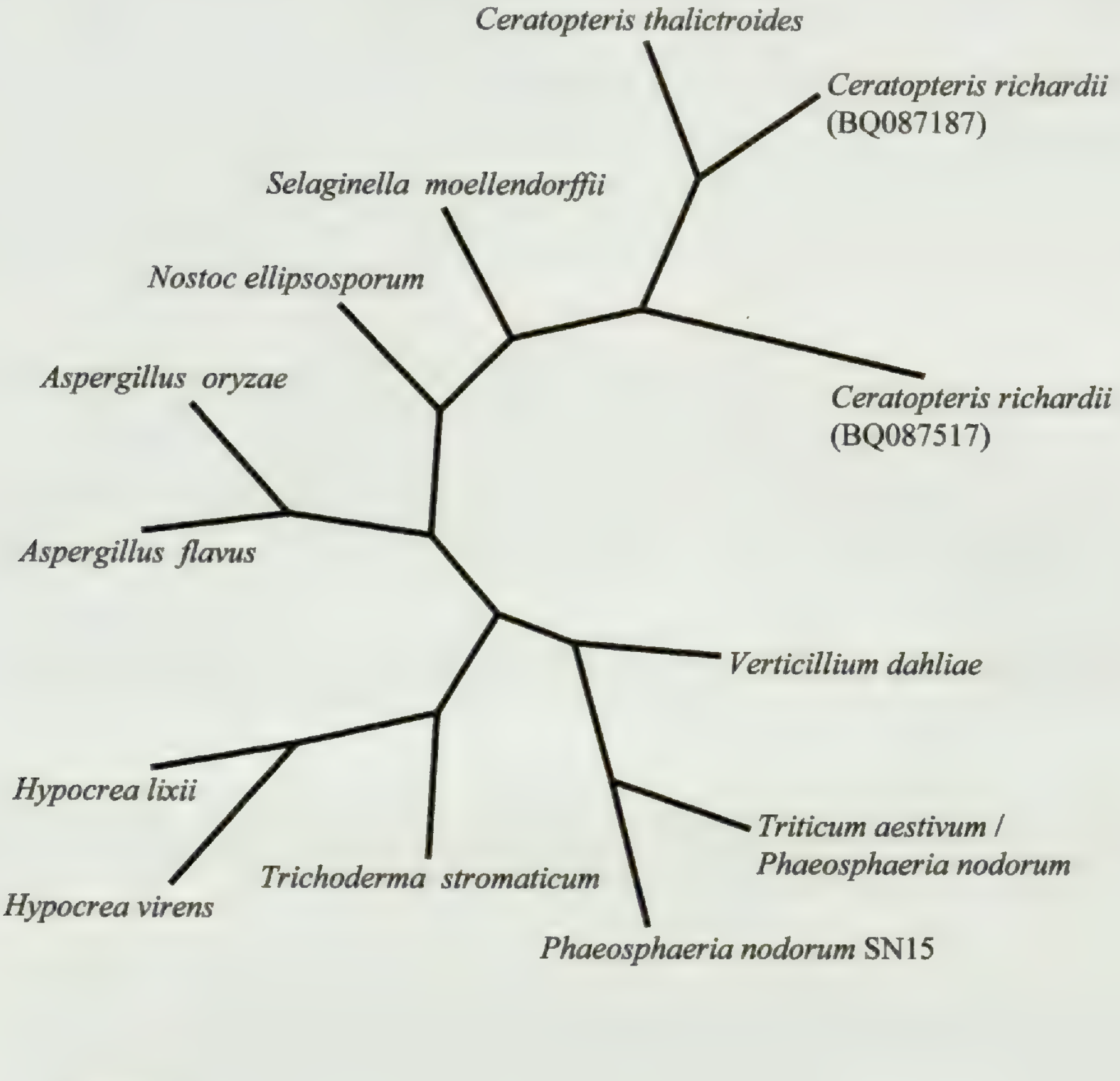


FIG. 6. Phylogeny of the CVNH proteins. The unrooted tree was constructed by neighbor-joining analysis (Saitou and Nei, 1987) of genetic distances estimated with the Jones-Taylor-Thornton model. Branch lengths are proportional to genetic distances as indicated by the scale bar representing 10 PAMs (point-accepted mutations).

belonging to different phyla form monophyletic groups. The CVNH domains may have common origin; however, Percudani et al. (2005) suggested that in fungi and seedless plants the domain has been separately amplified with different copy numbers following the separation of these two lineages.

Predicted CtCVNH tertiary structure and the structural evolution of CVNHs/CV-N.—Understanding the structural properties of CtCVNH is important for clarifying the conservation and variation of CVNHs as well as the roles they play in plants. *In silico* methods exist to predict with high reliability the tertiary structure of proteins from template structures (Saenz-Rivera *et al.*, 2004; Gopalasubramaniam *et al.*, 2008). Predicting a structure can yield insights into potential evolutionary patterns for CVNHs. Because CtCVNH and

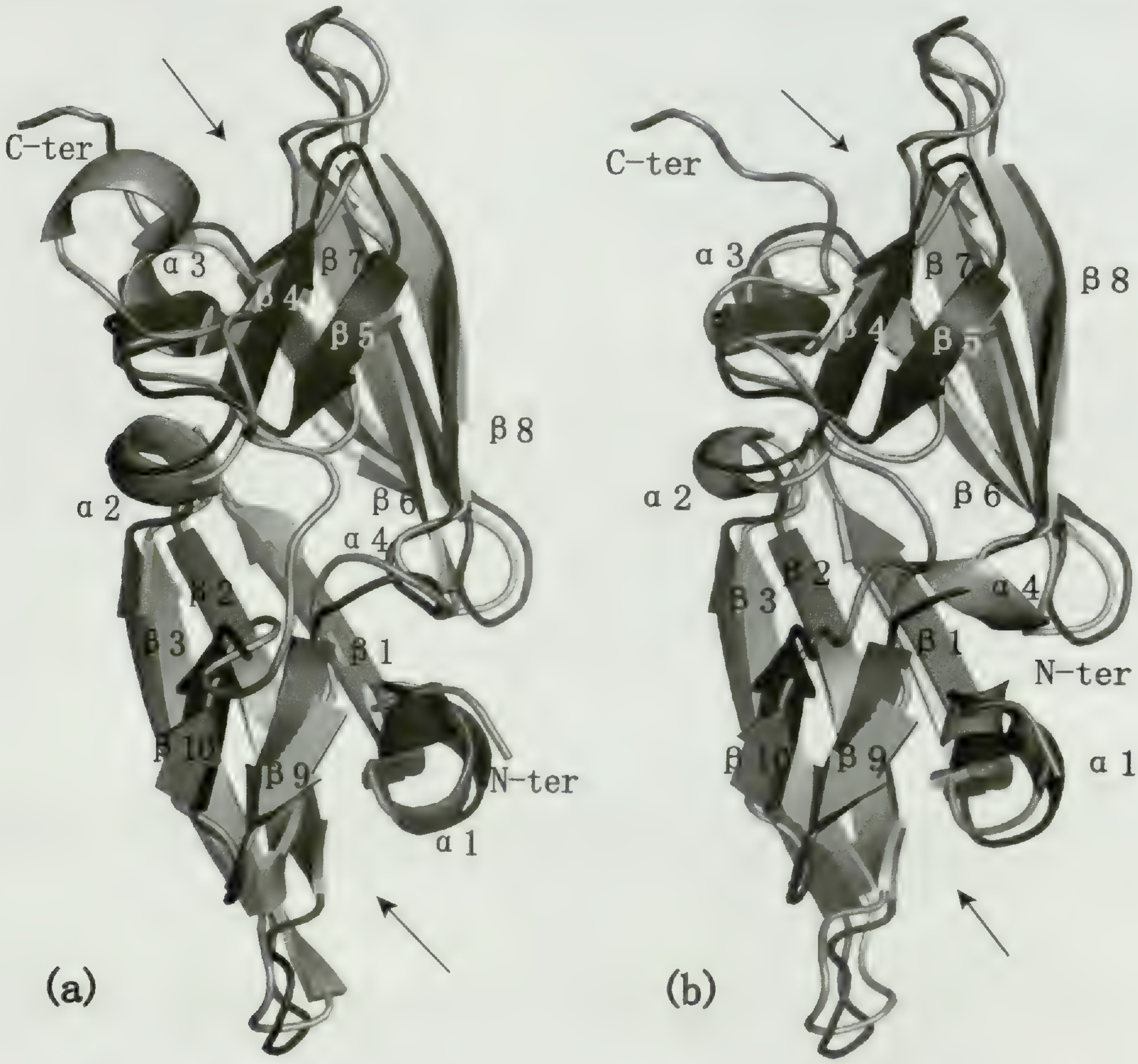


FIG. 7. Panel (a) overlay of predicted CtCVNH (gray) and native CV-N (black) tertiary structures. Panel (b) overlay of CrCVNH (gray) and native CV-N (black) tertiary structures. β-strands are indicated with β1–10 and helical turns with α1–4. Arrow shows the two sugar binding pockets of CV-N.

C. richardii CVNH (CrCVNH) are approximately 50% identical, we predicted the tertiary structure of CtCVNH using CrCVNH as a template. Fig. 7a further shows that the predicted CtCVNH comprises two tandem sequence repeats. They form equivalent, elongated structures via the combination of a triple-stranded β-sheet and a β-hairpin. Thus two symmetrically related fold-domains are created, each containing a sugar-binding site. Fig. 7a indicates that CtCVNH structure is quite similar to that of native CV-N, including the positions of triple-stranded antiparallel β-sheet (the first sequence repeat: β1, β2, and β3; the second: β6, β7, and β8), β-hairpin (formed by β4 and β5, β9 and β10, respectively), and α-helical turn (α1–4). However, the structures differ in that the N- and C-terminal regions are longer in CtCVNH than in CV-N, the helical turn (α3) folds differently, and an (3/4 turn) α-helix exists within the C-terminal region of predicted CtCVNH. Moreover, the β1 and β6 strands are

shorter in CtCVNH than in CV-N. To further understand the CVNH evolution in plants, we also compared the tertiary structure of CrCVNH with CV-N. Fig. 7b shows that the native CrCVNH structure is more similar to that of native CV-N, and most differences exist in the helical turn regions (α2, α3, α4) rather than in the β-strand ones. It is worthwhile to note that these differences are located in the sugar binding pockets of the proteins, which imply that CrCVNH and CV-N may have different affinities for mannose disaccharide ligands (Percudani *et al.*, 2005).

In conclusion, molecular cloning and characterization of CtCVNH showed that CtCVNH is very similar to other CVNHs from ascomycete fungi and the fern *C. richardii*, having a typical anti-HIV domain [Fig. 5(a), 7], indicating that CtCVNH belongs to CVNH family. This is the first time a full-length genomic DNA of CVNH in plants has been cloned. Our results provide a basis for a deeper understanding of CVNH function and evolution.

ACKNOWLEDGMENTS

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A New Species of *Adiantum* from Cuba

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ABSTRACT.—*Adiantum alomae* is described from eastern Cuba. It is characterized by pubescence on all parts of the leaves and its small size. Its habitat is also distinctive, occurring on limestone cliffs and walls, usually facing and very near the sea, receiving salt spray. A key is given to differentiate it from the related Cuban endemic *Adiantum sericeum*, and illustrations of the distinctive characteristics of both species are presented.

KEY WORDS.—*Adiantum alomae*, Cuba, new species

Adiantum is represented in Cuba by 23 species (Duek, 1971). These species grow mainly in gallery forest and secondary forest, from sea level to ca. 700 m in elevation or in coffee, cacao and citrus plantations. The genus is nearly absent in rain and cloud forests at high elevations.

Two species of *Adiantum* grow in eastern Cuba in coastal shrub vegetation or on limestone cliffs near or facing the sea. The first is *Adiantum alomae*, described below, which occurs mainly along the southern coast. The second is *A. deltoideum* Sw. occurring along the northern coast.

Eaton (1869) described *Adiantum sericeum* and pointed out that it differed from many other species of *Adiantum* by the pubescence on all parts of the leaf. Since then, many collections of densely pubescent *Adiantum* have been gathered in Cuba and identified as *A. sericeum*. Recent herbarium and field observations, however, suggest that they represent not one but two species: *A. sericeum*, growing in central and western Cuba, and a new one confined to eastern Cuba, which is here described.

Adiantum alomae Caluff, ***sp. nov.*** TYPE.—CUBA. Santiago de Cuba. Castillo del Morro y sus alrededores, en rocas y paredones calizos, localmente abundante, 0–50 m, matorral costero, 14 Jun 2007, *Caluff, Shelton, & M. Serguera 6356* (Holotype: BSC!; isotypes: HAC!, HAJB!). **Fig. 1 A–I.**

Ad *A. sericeum* D.C. Eaton affinis differt, frondium magnitudine (12–51 × 4–10 cm in *A. sericeo* autem 5–28 × 1.3–2 cm in *A. alomae*), laminae architectura (2–3 pinnata in *A. sericeo* autem 1–2 pinnata in *A. alomae*), rhizomatis squamarum longitudine (4–20 mm in *A. sericeo* autem 1.3–5 mm in *A. alomae*), atque rachium trichomatibus (trichomata densissima, rigida, patentia obscuro-rubescentiaque in *A. sericeo*, autem sparsa, flexuosa, albescentia usque claro-fusca in *A. alomae).*

Plants epipetric or rarely terrestrial. *Rhizome* ascending, ramified, cylindrical, blackish, 2–3 mm in diam., scaly toward the apex, the scales (Fig. 1E) ovate to ovate-lanceolate, brown, 1.3–5 × 0.8–1.2 mm, nearly clathrate, the

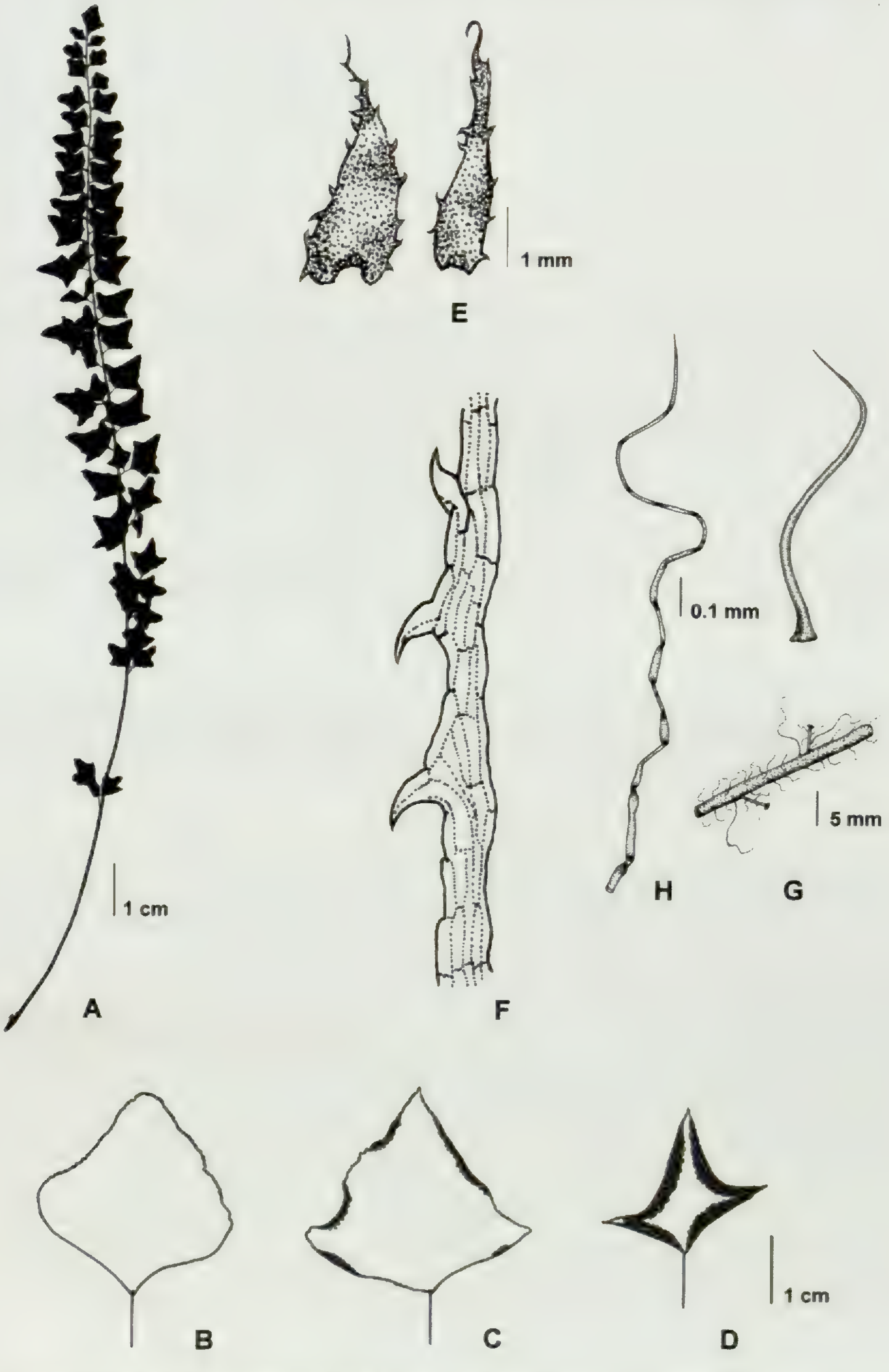

FIG. 1. *Adiantum alomae* Caluff. *Caluff & M. Serguera 6226* (BSC). A. Silhouette; B. Sterile apical pinnulae; C. Partially fertile apical pinnulae; D. Fertile apical pinnulae; E. Rhizome scales; F. Rhizome scale denticulations; G. Rachis and stalks pubescence; H. Rachis unicellular hair; I. Rachis pluricellular hair.

cells in the basal and medial portion roundish, distally enlarged, basifixed, the base truncate to more or less cordate, denticulate, the teeth (Fig. 1F) recurved or incurved, concolorous, usually conformed by two cells, the apex filiform: *fronds* (Fig. 1A) numerous, fasciculate, 5–30 cm long; *laminae* linear-lanceolate, 1-pinnate or occasionally 2-pinnate in the largest medial pinnae, 4–24 × 1–3.2 cm, with an apical, the biggest, conform pinna (Fig. 1B–D), usually hairy on both surfaces; *stipes* cylindrical, 1–6 cm long and 0.2–1.0 mm diam., reddish brown when young, eventually blackish, lustrous, scaly at the very base, hairy throughout like the rachis and pinnae stalks (Fig. 1G), the hairs weak and deciduous, of two types: some numerous, unicellular (Fig. 1I), whitish to clear brown, usually flexuous 0.8–0.9 (1.3) mm long, cylindrical, sometimes paler and flattish, with an enlarged base, others, occasional, found near the pinnae insertion and in the stalks, pluricellular (Fig. 1H), translucent, flattish, flexuous, with some cateniform cells, the septae reddish, up to 3 mm long, scales also present, these, deltate-enlarged, clear brown, lustrous, the base truncate, the cells 2–4 times longer than wide; *pinnae* 8–16 pairs, stalked 1–3 mm, 0.5–1.7 × 0.4–1.3 cm, alternate, with 3(–5) lobules, the margins entire to shallowly dentate, the base cuneate, lightly oblique, articulate, deciduous, the stalk and its blackish color stopping abruptly in a dilatate, discoid joint, the simple ones never overlapping the rachis, the sterile herbaceous, rounded or with blunt lobes, the fertile ones somewhat contracted, chartaceous, saggitate, the apex and lobules acute; *pinnules* (if any) 1 or 2, similar to the pinnae but smaller; *veins* free, flabellate-dichotomous, clear brown, lightly raised over the laminar tissue and hairy on both surfaces; *laminar hairs* abundant, unicellular, whitish, flexuous, 0.6–0.9 mm long. *Sori* linear, usually continuous and curved, avoiding the lobe apex; *indusia* brown to dark brown, entire, with numerous rigid, whitish to clear brown hairs, 0.4–0.5 mm long; *sporangia* glabrous; *spores* tan to yellowish, globose-tetrahedral, retate, 40–45 μm.

Specimens Examined.—CUBA. **Granma:** municipio Pilón, Boca de Toro, desembocadura del río Boca de Toro, suelo calizo, 0–10 m, 19 Mar 1988, *Gabriel Brull s/n* (HAC; HAJB). **Santiago de Cuba:** farallón costero, Sardinero, Santiago., 8 Jul 1949, *Alaín 815* (HAC); Oriente, Florida Blanca, sobre rocas, 10 Jan 1960, *Hno. Alaín, Acuña, López-Figueiras & Ramos s/n* (HAC); playa Sardinero, Justicí, Santiago de Cuba, entrando a la playa, sobre paredones calizos, 5 m, matorral xeromorfo costero, 13 Jul 1980, *Caluff 920* (BSC); Castillo del Morro de Santiago de Cuba, común en paredes y resquicios calizos, 50 msm, vegetación costera, 20 Aug 1979, *Caluff & Couso 1* (BSC); playa Sardinero, Reserva Siboney-Juticí, Santiago de Cuba, en rocas y paredones a 1 km de la playa, 10 m, bosque semidecíduo micrófilo, 29 Feb 2007, *Caluff & M. Serguera 6226* (BSC); paredones a la entrada de la playa Sardinero, Santiago de Cuba, 5 m, matorral xeromorfo costero, 19 Mar 1990, *Caluff & Shelton 2942* (BSC); alrededores de Santiago de Cuba, Jul 1920, *Clemente 131* (HAC); alrededores del Morro, Santiago, 18 Nov. 1937, *Clemente 2248* (HAC); sobre una roca en los farallones que rodean a la Playa de Sardinero, Santiago de

Cuba, 25 Nov 1951, *López Figueiras 315* (HAC); provincia de Oriente, cercanías de la desembocadura del río San Juan, en la playa de Aguadores, 26 Oct 1952, *López Figueiras 1716* (HAC, 3 sheets; HAJB); provincia de Oriente, en farallones del Monte Picote, cercanías del central Miranda, 12 Mar 1955, *López Figueiras 2001* (HAC; HAJB); Santiago de Cuba, Morro Castle, 24 7. 1909, *Voisard & Boelloz 1556* (HAC). **Without locality:** 27 Jul 1952, *F. G. C. s.n.* (HAC); *García Cañizares s.n* (HAJB); Cuba orientali, 1859–1860, *Wright 1078* (3 sheets) (HAC).

DISTRIBUTION.—Endemic to the coasts of southeastern Cuba, Santiago de Cuba and Granma Provinces, with a single small population found inland, growing in the karstic belt of the southern side of Sierra de Nipe Mountains.

HABITAT.—Terrestrial or epipetric in dry coastal shrubs vegetation and in semi deciduous microphillous forest, on limestone, in cave entrances, on big rocks, cliffs and old walls, exposed or partially shaded, usually in crevices, facing the sea and receiving the salt spray, 0–50 m elevation; the population inland, on limestone cliffs, usually in open and sunny places, 80–150 m elevation.

Adiantum alomae grows in dry habitats near and commonly facing the sea, receiving the salt spray. The largest population was found in the Morro Castle of Santiago de Cuba and nearby areas, consisting of hundreds of individuals. They grows on old walls, big rocks, around a cave entrance, in the ground. The stone masonry associated with the castle was built using calcareous rocks and terracotta bricks cemented with lime mortar.

Adiantum alomae and *A. sericeum* are the only two completely hairy species of this genus in Cuba. The hairs cover the stipes, rachises, indusia, and laminar tissue on both surfaces. These species can be distinguished by the following key:

1. Leaves up to 30 × 2.3 cm; lamina 1-pinnate to occasionally 2-pinnate; the simple pinnae not overlapping the rachis; unicellular hairs of the non laminar axes whitish to clear brown, weak, flexuous . . . . . ***A. alomae***
1. Leaves up to 51 × 10 cm; lamina 2-pinnate to occasionally 3-pinnate; basal acroscopic pinnule of each pinna overlapping the rachis; unicellular hairs of the non laminar axes dark reddish, acicular, rigid, patent . . . . . ***A. sericeum***

Adiantum alomae and *A. sericeum* resemble *A. tricholepis* Fée from the United States, Mexico, and Mesoamerica. They all have denticulate rhizome scales and pubescent laminar tissue on both surfaces. *Adiantum tricholepis* differs from the other two species by laminae ovate to deltate and 3–4 pinnate, the acroscopic basal pinnules not overlapping the main rachis, the rachis and costae glabrous, and the apex of the stalks not or lightly enlarged (Moran *et al.*, 1995).

Adiantum alomae is similar to *A. deltoideum* in habitat, small size, and pinna shape. *Adiantum deltoideum* differs basically in being glabrous and in its distribution, confined to the northern coast of eastern Cuba, growing likewise, on the northern and east coasts of Jamaica, and in Hispaniola (Proctor, 1985).

EPONYMY.—This species is dedicated to Omar Alomá Moreno, Director of the Macradenia Orchid Garden, in Palmira, Cienfuegos Province, central Cuba,

who first called my attention to the differences between *A. sericeum* and the new species.

Because the protologue for *Adiantum sericeum* is very simple and this rare Cuban endemic is poorly known, a complete description of this species is given.

Adiantum sericeum D. C. Eaton, Botanisch Zeitung. Berlin. 27. 361. 1869. TYPE.—prope Trinidad, *Wright 3950* (isotypes: MBG!; HAC!; NY! {5 sheets}). **Fig. 2 A–H**.

Plants epipetric or terrestrial. *Rhizome* ascending, branched, cylindrical, blackish, 2.5–9 mm in diam., densely scaly at the apex, the scales (Fig. 2E) lustrous, concolorous, light brown, deltate-attenuate to deltate-lanceolate, 4–10 × 0.3–1.5 mm, non clathrate, basifixed, the base cordate or lightly so, the margins denticulate (Fig. 2F) with spaced and usually straight, minute, concolorous, one or two celled teeth, the cells in the basal portion rounded to quadrangular, toward the medial and distal portion gradually more enlarged, 2–5 times longer than wide; *fronds* numerous (Fig. 2A), fasciculate, ca. 51 cm long; lamina 8–38 × 4–10 cm, lanceolate to oblanceolate, 2-pinnate throughout or occasionally 3-pinnate at the base and in the base of the medial largest pinnae, gradually tapered to an apical, conform, simple pinna similar to the apical pinnules of the largest pinnae (Fig. 2. B–D), herbaceous to papyraceous, densely hairy on both surfaces with unicellular, acicular, patent, whitish to deep reddish hairs 0.6–0.9 mm long; *stipes* 4–13 cm long and 0.6–1.8 mm diam., cylindrical, reddish black, lustrous, scaly at the very base with scales similar to thouse of the rhizome, densely hairy throughout like the rachises and stalks (Fig. 2G), the hairs deciduous, of two types, the commonest acicular (Fig. 2H), unicellular, patent, dark reddish, with a pustular, persistent, enlarged base, ca. 0.7 mm long, and very occasional hairs pluricellular, flexuous, some flattish, translucent with reddish septae, sometimes with some cateniform cells; *pinnae* 10–17 pairs, alternate, 2–7 × 1–2.7 cm, oblong-attenuate, stalked 2–5 mm, lightly oblique, with a terminal conform, biggest pinnule; *pinnules* alternate, oblique, articulate, stalked 1–3 mm, the black color of the stalk suddenly stopping at the pinnule base, leaving a discoid black joint when it falls off, the sterile ones larger, the base cuneate, distally rounded or with the apex and lobules blunt, the outer margins crenate and dentate to lobulate, the fertile ones contracted, saggitate, the apex and lobules acute, lobulate, dentate toward the lobules apex; *pinnules of the first order* 2–7 pairs, 0.5–1.7 × 0.5–1.7 cm, the basal acroscopic in each pinna overlapping the primary rachis, the apical one always the largest, ca. 2.1 × 2.3 cm; *pinnules of the second order* similar to those of the first order but more smaller; *veins* free, flabellate dichotomous, ending in teeth, light brown, lightly raised over the laminar tissue and hairy on both surfaces. *Sori* oblong, usually discontinuous, curved to straight, avoiding the lobe apex; *indusia* brown to dark brown, the margin entire, densely hairy with dark reddish, 0.4–0.5 mm long hairs; *spores* tan, globose-tetrahedral, lightly tuberculate.

FIG. 2. *Adiantum sericeum* D. C. Eaton. *Caluff, Shelton & O. Alomá 6224* (BSC). A. Silhouette; B. Sterile apical pinnulae; C. Partially fertile apical pinnulae; D. Fertile apical pinnulae; E. Rhizome scale; F. Rhizome scale denticulations; G. Rachis and stalks pubescence; H. Rachis unicellular hair.

SPECIMENS EXAMINED.—CUBA. **Sancti Spiritus:** Farallón del Charco de Oro, río Higuanojo, Area Protegida El Naranjal, Alturas de Sancti Spiritus, en farallones calizos, 300 m, 26 Aug 1994, *E. Bécqer & E. Martínez 3444* (BSC); alrededores del Hoyo del Naranjal, márgenes del río Higuanojo, Alturas de Sancti Spiritus, prov. Sancti Spiritus, en farallones rocosos, 280 m, bosque siempreverde secundario, 30 Nov 1994, *Caluff & Shelton 3854, 3855, 3856 A/B* (BSC): cascada Las Cortinas, arroyo La Yaba, finca La Vega, km 40 de la carretera desde Cienfuegos a Trinidad, a unos 200 m de la carretera, en un pedregal de rocas micáceas carbonatadas esquistosas, subiendo por el lado derecho de la cascada, 60–80 m, en bosque semidecíduo mesófilo, 3 Feb 2007, *Caluff, Shelton & O. Alomá 6224* (BSC, 16 sheets); **Cienfuegos:** arroyo Navarro, Mina Carlota, SE de Cumanayagua, Sierra de San Juan, 330 m., 22 Mar 1957, *Proctor 29409* (HAC). EASTERN CUBA. **Pinar del Río:** paredones del Pan de Azúcar, Viñales, del Río, 5 Feb 1956, *Acuña & Morton 20106* (HAC); sobre las rocas, base del mogote Pan de Azúcar, Viñales, 9 Oct 1955, *Alaín 4425* (HAC); La Guira, 7 km de Punta de La Sierra, Pinar del Río, exploración sur del mogote, 12 Nov 1972, *Bobrov & Cárdenas 29409* (HAC); exploración sur del mogote La Guira, 12 Nov. 1972, *Bobrov & Cárdenas 29811* (HAC); cercanías de Sumidero, Pinar del Río, Jul 1012, *J. A. Shafer & León 3171*, (*Shaffer 13407*) (HAC).

DISTRIBUTION.—Endemic to central Cuba, Sancti Spiritus, and Cienfuegos Provinces, Trinidad and Sancti Spiritus Heights, and western Cuba, Pinar del Río Province, Sierra de los Organos.

HABITAT.—Semi deciduous and evergreen secondary forest and in karstic (mogote) vegetation, on limestone, in well drained and inclined stony soil, on big rocks and cliffs, usually in crevices, in filtered sun, 60–300 m alt., locally common.

Adiantum sericeum grows inland in moderately humid places, typically with pteridophytes such as *Adiantopsis rupicola* Maxon, *Adiantum fragile* Sw., *Adiantum tenerum* Sw., *Anemia adiantifolia* (L.) Sw., *Anemia cuneata* Poepp. ex Spreng, *Blechnum occidentale* L., *Lygodium venustum* Sw., *Pteris longifolia* L., *Selaginella eatonii* Hieron. ex Small, *Selaginella* spp., *Thelypteris dissimulans* (Maxon & C. Chr.) C. F. Reed, *T. kunthii* (Desv.) C. V. Morton, and *T. scolopendrioides* (L.) Proctor.

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A New Brazilian Species of the Genus *Asplenium* L. (Aspleniaceae)

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ABSTRACT.—*Asplenium truncorum*, a new asplenioid fern from the Brazilian Atlantic Rain Forest, is described, illustrated and compared to the most similar species. So far, it seems to be restricted to the montane moist forests of southern Bahia and Espírito Santo States, at elevations of 750 to 950 m. Field observations suggest that this species grows exclusively as an epiphyte on the trunks of tree ferns, especially *Alsophila setosa* Kaulf. (Cyatheaceae).

KEY WORDS.—*Asplenium truncorum*, Atlantic Rain Forest, Bahia, Espírito Santo, ferns, taxonomy

The asplenioid ferns, including the genus *Asplenium* L. and its putative segregates, make up one of the most species-rich groups among leptosporangiate ferns, comprising approximately 700 species, mainly with tropical distribution (Schneider *et al.*, 2004; Smith *et al.*, 2006). According to Sylvestre and Windisch (2003), Brazil harbors about 70 species of *Asplenium*, representing nearly half of the diversity found in the Neotropics (close to 150 species, according to Tryon and Tryon, 1982). As is the case with many other fern genera (*e.g.*, Moran, 1981; Moran *et al.*, in press), the Serra do Mar mountains along the coast of southeastern Brazil play a very important role in the diversification of this group, presenting a high level of endemism. Recent botanical expeditions to these mountains, in the States of Bahia and Espírito Santo, have revealed a new species of the genus *Asplenium*, which we describe as follows:

Asplenium truncorum F. B. Matos, Labiak & L. Sylvestre, ***sp. nov.*** TYPE.—BRAZIL. **Bahia**: Camacan, RPPN Serra Bonita, 15°23′25″S, 39°34′05″W, 920 m, 29 Jul 2008, *F. B. Matos et al. 1537* (holotype: UPCB; isotypes: CEPEC, NY, RB, SP). **Figs. 1, 2A–C, F–G.**

Species *Asplenio martiano* C. Chr. similaris, differt laminis minus divisis, ad basin 1-pinnatis, petiolis laminis dimidio brevioribus et habitu epiphytico.

Plants epiphytic. *Rhizomes* erect; *scales* 1.5–2 × 0.3–0.5 mm, lanceolate, atrocastaneous, clathrate, tips twisted and long attenuate, margins with

FIG. 1. A–D. *Asplenium truncorum* (*Matos 1537*, UPCB). A. Habit. B. Medial portion of the lamina. C. Rachis and sori detail. D. Rhizome scales.

irregular projections; *roots* thin and wiry, not proliferous. *Fronds* (5)10–16(30) cm long, arcuate, clustered; *indument* abaxially of scattered, linear, clathrate scales, 0.2–1 mm long, also with inconspicuous clavate hairs, especially on leaf axes. *Stipes* 2–5(8) cm long, 0.3–0.7 mm diam., ca. 1/3–1/2 of the lamina length, brownish at base and greenish to stramineous distally, dull, with narrow green wings less than 0.4 mm wide. *Blades* 4–15(21) cm long × 1–5(13)

FIG. 2. A–C. Silhouettes illustrating the morphological variation in *Asplenium truncorum*. A. (*Matos 1537*, MBM). B. (*Matos et al. 806*, CEPEC). C. (*Matos 1537*, UPCB). D–E. *Asplenium martianum* (*Handro 2664*, NY), spores with alate folds, echinulate wings. D. Lateral view of the spore. E. Distal view of the spore. F–G. *A. truncorum* (*Thomas et al. 13796*, CEPEC), spores with low folds and large areolas. F. Lateral view of the spore. G. Proximal and lateral view of the spores.

cm wide, membranaceous, 1-pinnate proximally, with long attenuate pinnatifid apices; *rachises* greenish to stramineous, dull, with narrow green wings up to 0.5 mm wide; *pinnae* 1–11 cm long, less than 1 cm wide, flabellate to linear-lanceate, subfalcate, 2–5 pairs, the bases cuneate, non-auriculate, apices obtuse

to long-attenuate, margins dentate; *veins* mostly simple except for the proximal ones, which are forked, readily visible on both sides, vein ends expanded adaxially. *Sori* 1–5(12) pairs per pinna, occasionally diplazioid; *indusia* 5 mm long × 0.3 mm wide, linear, firmly membranaceous, margins entire; *spores* reniform, monolete, with a few large and broad anastomosing ridges.

DISTRIBUTION AND ECOLOGY.—*Asplenium truncorum* is known only from the montane moist forests of coastal Brazil, in the States of Bahia and Espírito Santo, at 750–950 m above sea level. This species seems to grow exclusively as a low-trunk epiphyte on tree ferns (Fig. 1, A), especially *Alsophila setosa* Kaulf. (Cyatheaceae).

CONSERVATION.—Despite of its remarkable species richness and exceptional concentration of endemics, the devastation of the Brazilian Atlantic Forest continues at a very alarming rate. Nowadays it is considered one of the most threatened biomes on Earth, with a very fragmented distribution along the Brazilian coast. Because it has a narrow extent of occurrence in this scenario, *Asplenium truncorum* meets the IUCN criteria (IUCN, 2001) of vulnerable species (VU: B1 a + b iii).

ETYMOLOGY.—The specific epithet "truncorum" was chosen due to its habitat preference for tree fern trunks.

PARATYPES.—BRAZIL. **Bahia:** Almadina, Serra do Corcovado, 9,8 km ao SW de Coarací na estrada para Almadina, daí N até a Fazenda São José, 14°42′21″S, 39°36′12″W, 750 m, 19 Apr 2007, *Matos et al. 1408* (CEPEC, UPCB); Camacan, RPPN Serra Bonita, 15°23′30″S, 39°33′55″W, 835 m, 1 Feb 2004, *Thomas et al. 13796* (CEPEC); Camacan, RPPN Serra Bonita, 15°23′30″S, 39°33′55″W, 835 m, 3 Feb 2005, *Matos et al. 305* (CEPEC, UPCB); Camacan, RPPN Serra Bonita, 15°23′30″S, 39°33′55″W, 835 m, 13 Feb 2005, *Matos et al. 446* (CEPEC, UPCB); Jussari, RPPN Serra do Teimoso [750 m], 27 Jul 2005, *Matos et al. 806* (CEPEC, UPCB). **Espírito Santo:** Santa Teresa, Alto São Lourenço, Sítio da Cachoeira, 25 Oct 2000, *Demuner et al.* 1477 (BHCB, MBML); Santa Teresa, Nova Lombardia, Reserva Biológica Augusto Ruschi [800 m], 27 Jul 2002, *Vervloet et al.* 559 (BHCB, MBML); Santa Teresa, Nova Lombardia, Reserva Biológica Augusto Ruschi [800 m], 18 Dec 2002, *Rose & Pereira 20* (BHCB, MBML).

Asplenium truncorum can be recognized by its erect rhizome, stipes with ca. 1/3 to 1/2 of the lamina length, 1-pinnate or less divided lamina, non-conform apical pinnae, and membranaceous to chartaceous leaf texture. Superficially, it resembles *Asplenium auriculatum* Sw. in habit, leaf dissection and color. However, the latter can be easily recognized by the presence of prominent auricles in the acroscopic base of the pinnae that often overlap the rachis. *Asplenium martianum* C. Chr. is probably one of the most closely related species in Brazil, being distinct by its longer stipes (the same length as the lamina or longer), blades usually 2-pinnate at base (or at least deeply 1-pinnate-pinatifid), and preferentially terrestrial habitat. Besides that, their spores are quite distinct, with those of *Asplenium martianum* showing alate

folds and echinulate wings (Fig. 2, D–E). *Asplenium austrobrasiliense* (Christ) Maxon also seems to be related morphologically, differing mainly by its chartaceous to coriaceous blades with conform apical pinnae, and longer stipes with approximately the same length as the laminae. *Asplenium cariocanum* Brade, which is ecologically similar in habitat, differs in having fringed stem scales with pronounced dark teeth, pinnae with lobately serrate margins and nearly symmetric pinnae bases that are usually auriculate.

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New Combinations in *Pleopeltis* (Polypodiaceae) from Southeastern Brazil

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ABSTRACT.—From taxonomic studies of *Pleopeltis* from southeastern Brazil, some new combinations are made: *Pleopeltis alborufula* (Brade) Salino, *P. bradei* (de la Sota) Salino, *P. desvauxii* (Klotzsch) Salino, *P. minarum* (Weath.) Salino, *P. monoides* (Weath.) Salino, and *P. trindadensis* (Brade) Salino.

KEY WORDS.—Ferns, pteridophytes, Polypodiaceae, *Pleopeltis*, Southeastern Brazil

The generic limits of *Pleopeltis* are under active revision (Andrews and Windham, 1993; Windham, 1993; Sota, 2003; Schneider *et al.*, 2004; Schneider *et al.*, unpubl. ms.). The most recent definition of *Pleopeltis* based on molecular phylogeny (Schneider *et al.*, 2004) includes the genera *Dicranoglossum* and *Neurodium*, as well as the *Polypodium* species with scaly leaf blades. The distribution of scales in these squamate species varies widely, but at least some are always present between the veins on the abaxial side of the blade (Kessler and Smith, 2005). In this definition, *Pleopeltis* comprises about 75 Neotropical and a few African species (Kessler and Smith, 2005). Some of the necessary combinations in *Pleopeltis* for Brazilian squamate *Polypodium* have been made by Sota (2003), Kessler and Smith (2005) and Sota *et al.* in Zuloaga *et al.* (2007), but other *Polypodium* and *Dicranoglossum* species need to be transferred. The necessary new combinations are proposed here to allow their use in regional floras and modern taxonomic treatments. The *Polypodium* species combined here were studied by Weatherby (1947) and Sota (1965, 1966) and clearly belong to the squamate clade of Schneider *et al.* (2004). With these additions, *Pleopeltis* is represented in Brazil by 14 species, with seven endemic to the southeastern region.

NEW COMBINATIONS

Pleopeltis alborufula (Brade) Salino, ***comb. nov.*** *Polypodium alborufulum* Brade, Arq. Jard. Bot. Rio de Janeiro 11: 29. 1951. TYPE.—BRAZIL, Espírito Santo, Castelo, Forno Grande, 12 May 1949, *A.C. Brade 19791* (RB !).

DISTRIBUTION.—Endemic to Brazil (only in Espírito Santo state).

Pleopeltis bradei (de la Sota) Salino, ***comb. nov.*** *Polypodium bradei* de La Sota, Revista Mus. La Plata, Secc. Bot. 9 (42): 266. 1965. TYPE.—BRAZIL,

Espírito Santo, Castelo, Forno Grande, 12 May 1949, *A.C. Brade 19791 B* (RB!).

DISTRIBUTION.—Endemic to Brazil (only in Espírito Santo state).

Pleopeltis desvauxii (Klotzsch) Salino, ***comb. nov.*** *Taenitis desvauxii* Klotzsch, Linnaea 20: 431. 1847. LECTOTYPE.—(designated by Proctor, Flora Lesser Antiles 348. 1977): Hooker & Greville, Icon. Fil. 1, t.7. 1827, based on a Guilding specimen from St. Vincent (not seen).

DISTRIBUTION.—Neotropical.

Pleopeltis minarum (Weath.) Salino, ***comb. nov.*** *Polypodium minarum* Weath., Contr. Gray Herb. 165: 78. 1947. TYPE.—BRAZIL, Minas Gerais, Serra da Piedade, *1843. Claussen 78* (P, not seen).

DISTRIBUTION.—Endemic to Espinhaço range and Iron Quadrangule, in Minas Gerais state, Brazil.

Pleopeltis monoides (Weath.) Salino, ***comb. nov.*** *Polypodium monoides* Weath., Contr. Gray Herb. 165: 78. 1947. TYPE.—BRAZIL, Bahia, forests of the rio Gongogi basin, 100–300 m, 10/30 Nov. 1915 (US!).

DISTRIBUTION.—Endemic to Brazil (Bahia, Espírito Santo, and Minas Gerais states).

Pleopeltis trindadensis (Brade) Salino, ***comb. nov.*** *Polypodium trindadense* Brade, Arq. Inst. Biol. Veg. 3: 4, t. 3, 4, 6. 1936. TYPE.–BRAZIL, Ilha da Trindade, 14 Jan. 1917, *C. Porto 585* (RB!).

DISTRIBUTION.—Endemic to Trindade Island, Brazil.

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A Hybrid *Phlebodium* (Polypodiaceae, Polypodiophyta) and Its Influence on the Circumscription of the Genus

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Abstract.—The fern genus *Phlebodium* is traditionally described as having a row of costal areoles lacking included veins, with the sori located in extra-costal areoles and each sorus served by two veinlets. The discovery of a hybrid between *Phlebodium pseudoaureum* and *Polypodium pleurosorum* raises questions about the limits of *Phlebodium* and necessitates a revised taxonomic circumscription of the genus.

Key Words.—ferns, hybrid, Mexico, *Phlebodium*

The fern genus *Phlebodium* has a Neotropical distribution and has been thought to comprise three species: *P. aureum* (L.) J. Sm., *P. decumanum* (Willd.) J. Sm., and *P. pseudoaureum* (Cav.) Lellinger [syn. *P. areolatum* (Humb. & Bonpl. ex Willd.) J. Sm.] (see e.g., Proctor, 1989; Nauman, 1993; Mickel and Smith, 2004). When first recognized at generic rank, *Phlebodium* (R. Br.) J. Sm., based on *Polypodium* sect. *Phlebodium* R. Br., was a superfluous name because it included sect. *Pleopeltis* Humb. & Bonpl. ex Willd., an older name that should have been adopted under current rules (see Smith, 1981). Article 52.3 (McNeill *et al.*, 2005; see also its Ex. 15) is applicable to this matter. Since *Phlebodium* is based on a name-bringing synonym (in other words, it has a basionym, i.e., *Polypodium* sect. *Phlebodium* R. Br., that is legitimate), *Phlebodium* is not illegitimate. Because Smith's genus was a stat. nov., Art. 7.4 dictates that the type of R. Brown's section must also be the type of *Phlebodium*. Art. 10.2 establishes that the type must be either *P. aureum* or *P. decumanum*, given that these were the only two species included in sect. *Phlebodium* by Brown. *Phlebodium* was lectotypified by *Phlebodium aureum* (L.) J. Sm. (Smith, 1875), and this choice has been reaffirmed by several authorities (e.g., Copeland, 1947; Tryon and Tryon, 1982).

Phlebodium has usually been characterized by venation that is highly reticulate (but free near margins), with 1 to 4 rows of fertile costal polygonal areoles and two or three rows of alternate marginal sterile areoles (without free included veinlets) (Fig. 1J). The costal areoles include one secondary areole that extends laterally from secondary vein to secondary vein, with two

included excurrent veinlets meeting at apices. The genus is further characterized by having pinnatifid to pinnatisect blades (Fig. 1G).

Often, *Phlebodium aureum* has been treated in a broad sense (e.g., by Tryon and Stolze, 1993), to include also *Ph. pseudoaureum* and segregates of that species. Tryon and Tryon (1982) placed *Phlebodium aureum* s.l. and *Polypodium lowei* C. Chr. [= *Po. pleurosorum*] in with a group of Mexican and Mesoamerican species related to *Polypodium plesiosorum* Kunze, *P. subpetiolatum* Hook., and several other species. The *Po. plesiosorum* group is now thought to be closely related to true *Polypodium* (type: *Po. vulgare* L.), and less closely related to *Phlebodium* (Schneider *et al.*, 2006; Tejero-Díez, 2005).

In 2002, the first author (JDTD) discovered in Chiapas, Mexico, a specimen (Fig. 1 A–C) that appears to be a hybrid between the most common species of *Phlebodium* in Mexico, *Ph. pseudoaureum* (Figs. 1G–J), and a simply pinnate species of *Polypodium*, *Po. pleurosorum* Kunze ex Mett. (Figs. 1D–F). The plant has blades that are pinnate proximally and pinnatifid distally, a mixture of sori each served by a single vein or by two veins, and differential development of secondary costal sterile areoles (Figs. 1A and C). Its sori have abundant sporangia and mostly malformed spores (Fig. 2H). Some authorities have considered *Phlebodium* and *Polypodium* as only distantly related (e.g., Copeland, 1947, who thought *Phlebodium* to be derived from *Pleopeltis*), while others have thought them to be more intimately related (e.g., Tryon and Tryon, 1982, p. 691). Closer examination was made to see if *Polypodium pleurosorum* might in fact belong to *Phlebodium*. Moore (1855), in his description of *Polypodium pleurosorum* (under the name *Phlebodium inaequale* T. Moore) wrote: "The sori are large, round, situated in a single series near the midrib; sometimes seated on the apex of a veinlet within a costal areole, which is characteristic of *Goniophlebium*; sometimes on a veinlet exterior to the costal areole, sometimes at the point where two or more veins unite, which is the normal condition of *Phlebodium*. It is consequently an osculating species between the genus *Goniophlebium* and *Phlebodium*." He also noted that it resembles *Phlebodium aureum* but has truly pinnate fronds. Examination of herbarium specimens of *Polypodium pleurosorum* shows that although most of the sori are located in costal areoles and served by a single vein, there are occasional sori, especially distally, that are served by two veins.

Recent phylogenetic studies based on DNA molecular characters (Schneider *et al.*, 2004; Schuettpelz and Pryer, 2007) show that *Phlebodium pseudoaureum* and *P. decumanum* are sister to a clade comprising species of *Pecluma*. Sampled are *Pe. alfredii* (Rosenst.) M. G. Price, *Pe. eurybasis* (C. Chr.) M. G. Price, and *Pe. ptilodon* (Kunze) M. G. Price and two Mexican/Mesoamerican species of *Polypodium*, *Po. hartwegianum* Hook. and *Po. longepinnulatum* E. Fourn. the last two species, as well as some others, are probably better referred to *Pecluma*, but these transfers await more comprehensive sampling in the *Pecluma* clade. The *Phlebodium* + *Pecluma* clade is in turn sister to a large group (75+ spp.) of scaly polypods, the *Pleopeltis* clade (Otto *et al.*, in press), including scaly species usually included in *Polypodium* s.l. The true *Polypodium* clade, comprising *Po. vulgare* L. and allies (Haufler and Ranker,

FIG. 1. A–C. *Phlebodium ×hemipinnatum* (*Tejero-Díez 4362*, NY). A. Habit. B. Rhizome scale. C. Pinna detail. D–F. *Phlebodium inaequale* (*Mickel 1099*, NY). D. Rhizome and pinnae. E. Rhizome scale. F. Pinna detail. G–J. *Phlebodium pseudoaureum* (*King & Soderstrom 4757*, MICH). G. Rhizome and blade. H. Rhizome scale. J. Pinna detail.

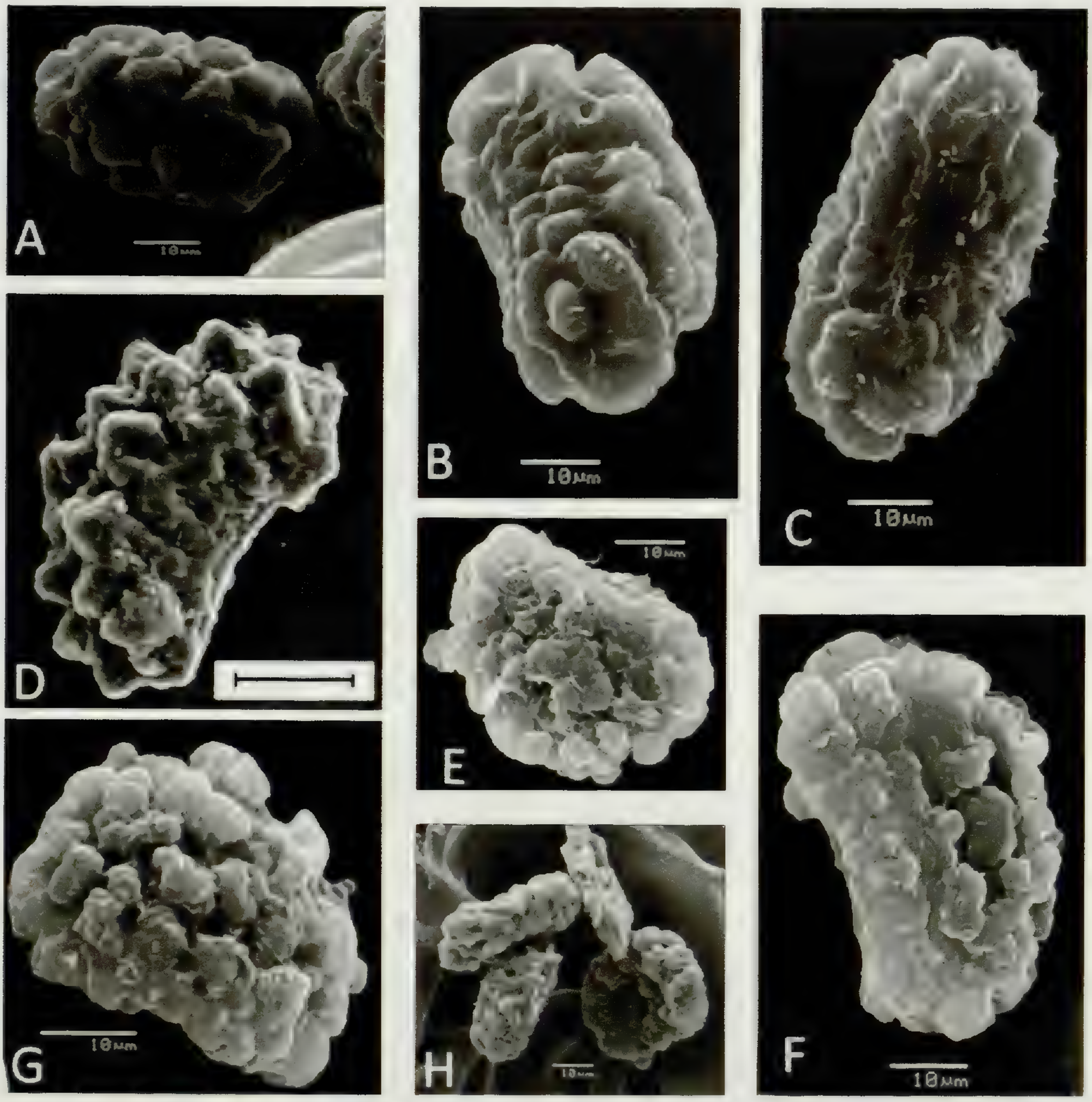

FIG. 2. Spores of *Phlebodium*. A–C. *Phlebodium inaequale* (*Tejero-Díez 4931*, IZTA). A. Distal view. B. Medial view. C. Proximal view. D. *Phlebodium pseudoaureum* (*Tejero-Díez 4195*, IZTA). Medial view (bar 20 µm). E–H. *Phlebodium* ×*hemipinnatum* (*Tejero-Díez 4362*, MEXU). E. Medial view. F, G. Proximo-medial view. H. Malformed spores.

1995), is yet more distantly related to *Phlebodium*. *Phlebodium inaequale* has now also been sampled for DNA (Schneider, unpubl. data), and nucleotide sequence data show that *Phlebodium*, as redefined here and including the newly transferred *P. inaequale*, is monophyletic, with strong bootstrap and Bayesian support, sister to the *Pecluma* alliance (Schneider, pers. comm.)

Proctor (1989) reported that in Puerto Rico, where *Phlebodium aureum*, *Ph. pseudoaureum*, and *Ph. decumanum* occur together, both *P. pseudoaureum* and *P. decumanum* appear to be diploid, *Phlebodium aureum* s.s. is their fertile, allotetraploid hybrid, and at least one sterile, triploid backcross hybrid was reported. Chromosome counts for *Phlebodium* include three counts of $2n = 74$ (diploid, based on $x = 37$) for *Ph. decumanum* from Trinidad (Walker,

1985), three counts of $n = 74$, $2n = 147$ for *Ph. aureum* from Trinidad and Tobago (Walker, 1985), and four counts $n = 37$, 2n = 74 of *Ph. aureum* s.l. from Jamaica and Mexico (Walker, 1966; Mickel and Smith, 1977, reported as *Po. araneosum* M. Martens & Galeotti, now considered a synonym of *Ph. pseudoaureum*). These last diploid counts likely pertain to the species now called *Ph. pseudoaureum*, and not the true *Ph. aureum*, which appears to be tetraploid. Walker (1985) reported spontaneous, sterile, triploid hybrids between what he called *Po. aureum* s.l. and *Po. decumanum* in Trinidad. There is also an early report of a hybrid called *Phlebodium* ×*schneideri*, reputed to be the hybrid between *Po. aureum* s.l. and *Po. vulgare* L. (Schneider, 1894). The parentage of this hybrid now seems in doubt, because of the relatively distant relationship between *Phlebodium* and *Polypodium*, as currently defined.

In an attempt to verify hypothesized relationships among species of *Phlebodium*, Caruso (1985) studied living plants of *Phlebodium aureum*, *Ph. pseudoaureum*, and *Ph. decumanum* growing in the greenhouses of the New York Botanical Garden. Although cytological studies were unsuccessful, measurements of spores and stomatal guard cells showed significant differences, with the tetraploid, *Ph. aureum* having the larger measurements.

The rarity of the Tejero-Díez collection (*4362*) and its morphological intermediacy suggest that it is a hybrid, and with its significant bearing on the circumscription of the genus *Phlebodium*, we hereby give it a hybrid name.

Phlebodium ×***hemipinnatum*** Tejero, Mickel and A. R. Smith, **hyb. nov.** TYPE.—MEXICO: **Chiapas**, Mpio. San Cristóbal de las Casas, Km 67 de la carretera federal 190, Tuxtla Gutiérrez a San Cristóbal de las Casas (16° 42′ 23″ N, 92° 46′ W), bosque de *Pinus-Quercus*, 2440 m, 6 Ago 2002, *Tejero-Díez 4362* (Holotype: MEXU; isotypes: IEB, IZTA, NY, UAMIZ). **Figs. 1A–C.**

Phlebodio pseudoaureo atque *Polypodio pleurosoro* proxima, sed laminis hemipinnatis, id est basis pinnatis apiceque pinnatifidis, plane differt.

Rhizomes long-creeping, 4–6 mm diam. (excluding scales), pruinose, densely scaly; *rhizome scales* 8–12 × 2–4 mm, ovate, long-attenuate, yellowish brown, each with enlarged, round, peltate base, darker at point of attachment, margins denticulate to short-ciliate and erose throughout, with short to long, flexuous, contorted, hairlike tips; *fronds* (55) 60–70 cm long; *stipes* 1/3–1/2 the frond length, brown, glabrous; *blades* ovate-deltate to broadly-oblong, 26–35 cm wide, 1-pinnate at middle basal part, becoming pinnatifid above the middle, terminal segment subconform, 5–16 cm long; pinnae (segments) 8–12 pairs, 12–30 mm wide, linear-oblong to linear-lanceolate, some falcate, acuminate, glabrous, green-yellowish, margins entire to repand; *veins* netted, free near margins, with 1 row of fertile costal polygonal areoles, each with a single simple or bifurcate, excurrent included veinlet or 2 veinlets that form a secondary areole and meet at their tips, 2–3 rows of similar areoles closer to pinna margins, these mostly without included veinlets; *sori* round, 2–3 mm diam., submedial, one row on each side of the costa; *spores* mostly malformed,

bilateral, monolete, (33)39(45) × (22)26(33) μm, tuberculate, tubercles dome-shaped, somewhat overlapping, amber.

PARATYPE.—MEXICO: **Chiapas**, Mpio. Tenejapa, a 3.5 km al NE del paraje Balum Canal (16° 48′ 05″ N, 92° 31′ 50″ W), Acahual derivado de bosque de *Pinus-Quercus*, 2200 m, 8 Mar 1995, *Ramírez-Marcial & Hernández-Rojas 654* (MEXU!, ECOSUR - herbarium of the Colegio de la Frontera Sur, Chetmul, Quintana Roo, Mexico).

HABITAT.—Epiphytic in pine-oak forests and adjacent disturbed areas; 2200–2500 m.

DISTRIBUTION.—Mexico, Chiapas, montane areas.

The existence of this new hybrid, with characters intermediate between *Phlebodium pseudoaureum* and *Polypodium pleurosorum*, causes us to conclude that the latter species can once again be included in the genus *Phlebodium*, with the earliest available name, *Ph. inaequale* T. Moore. Impetus for the recircumscription of polypod genera has been given by several other recent phylogenetic studies on Polypodiaceae, most importantly the one by Schneider *et al.* (2004), outlining a global phylogeny for the family. Subsequently, several other papers directed toward the placement of problematic Neotropical polypods have appeared (e.g., Krier *et al.*, 2007; Schneider *et al.*, 2006; Tejero-Díez, 2005), are in press (Krier *et al.*, 2008), or have been submitted for publication (Otto *et al.*, in press). The redefinition of *Phlebodium* also recalls the recent recircumscription of the polypod genus *Microgramma*, necessitated by the finding of a new and radically different species of the genus in coastal Brazil (Salino *et al.*, in press).

Cladistic analysis of morphological characters in species of *Polypodium* and related taxa (Tejero-Díez, 2005) suggests that the critical characters separating *Phlebodium* from its sister group (*Pecluma*, and Mexican/Mesoamerican species allied to *Pecluma* but still placed in *Polypodium*; Schneider *et al.*, 2004; Schuettpelz and Pryer, 2007) are: a) spores with tuberculate ornamentation (Fig. 2A–H; b) small size of spore body (33) 38 (45) μm; and c) the presence of several rows of marginal sterile polygonal areoles.

Of the aforementioned characters, the spore ornamentation in *Phlebodium* and the smaller spore size are unique in Polypodiaceae, but the ornamentation is somewhat similar to spores of *Polypodium arcanum* Maxon and some species of *Serpocaulon* (Tryon and Lugardon, 1991; Tejero-Díez, 2005). It is clear that the taxonomic limits of *Phlebodium* cannot be governed by the way in which the internal veinlets of the main costal areoles are organized.

The species of *Phlebodium* and the newly described hybrid can be separated by the following key:

1. Blades 1-pinnate, at least proximally; sori each at the end of a simple or bifurcate veinlet; secondary costal areoles absent or irregularly so.
   2. Blades pinnate throughout their length. . . . . *P. inaequale*
   2. Blades pinnate proximally but pinnatisect or pinnatifid distally. . . . . *P. ×hemipinnatum*
1. Blades pinnatifid or pinnatisect; sori each at the end of two veinlets; secondary costal areoles regularly present.

3. Sori in 1 row on each side of costae; (170–)550–2500 m. . . . . *P. pseudoaureum*
3. Sori in 2 or more rows on each side of costae; 0–500 m.
   4. Sori on 3 or more rows on each side of costae. . . . . *P. decumanum*
   4. Sori on 2 (infrequently 1) rows on each side of costae.. . . . . *P. aureum*

The use of the name *Phlebodium inaequale* T. Moore for what has been called *Polypodium pleurosorum* Kunze ex Mett. requires a brief explanation. The former name was published first by Moore (1855), but when treated as belonging in *Polypodium* cannot be used because of the existence of an earlier homonym, *Polypodium inaequale* Link, published in 1833 (Mickel and Smith, 2004).

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We thank Harald Schneider for permission to use unpublished information on the phylogenetic relationships of *Phlebodium inaequale*. We also thank John Wiersema for nomenclatural advice on the legitimacy of *Phlebodium*. Spore images were obtained by Rafael Emiliano Quintanar-Zuñiga, using a scanning microscope Jeol 6380 LW at the Facultad de Estudios Superiores Iztacala of the Universidad Nacional Autónoma de México. Haruto Fukuda prepared the line drawings in Fig. 1.

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American Fern Journal 99(2):117–141 (2009)

2008 AFS Symposium Summary

Summary of the 2008 AFS Symposium: From Gels to Genomics: The Evolving Landscape of Pteridology. A Celebration of Gerald Gastony's Contributions to Fern Evolutionary Biology.—The study of pteridophyte evolutionary biology has undergone remarkable developments during the past 40 years. Central to these developments have been the efforts of Gerald J. Gastony and his academic offspring to advance our understanding of these plants. Accordingly, on 29 July, 2008, during the Botany 2008 Conference in Vancouver, British Columbia, former students, colleagues, and friends gathered to celebrate Jerry Gastony's productive career at the forefront of pteridology. The symposium highlighted some of the major methodological and philosophical advances that have evolved during his exemplary career. Trained as a classical taxonomist, Prof. Gastony has continually reinvented himself since arriving at Indiana University in 1970. His initial forays into enzyme electrophoresis shed light on such diverse topics as the breeding system of ferns, the role of cryptic taxa in reticulate lineages, and the contributions of paleo- and neopolyploidy to fern systematics and evolution. These questions have been persistent throughout Jerry's career and have been influential in shaping the field of pteridophyte evolutionary biology. The 2008 AFS symposium revisited these questions and showed how new tools are building on the foundation that Prof. Gastony helped lay over the last 40 years. In lieu of a formal Proceedings, the present text presents a brief summary of each of the presentations from the symposium, credited individually to each speaker and his co-authors.—Edited by Michael S. Barker, Department of Botany, University of British Columbia, 3529-6270 University Blvd, Vancouver, BC V6T 1Z4, CANADA, and Department of Biology, Indiana University Jordan Hall 142, 1001 E Third St., Bloomington, IN 46405-3700 and George Yatskievych, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299.

A Brief History of Gerald J. Gastony's Botanical Career.—After graduating from St. Ignatius High School in Cleveland, Ohio, Gerald J. Gastony (1940–) attended St. Louis University for his undergraduate training. His initial focus was on the humanities and in 1964 he received his Bachelor's Degree in the College of Philosophy and Letters. Through this focus, he became fluent in Latin and comfortable in Greek, skills that aided his future career as a plant systematist. Jerry also became interested in botany through a course from the distinguished taxonomist and floristician, John Dwyer, and he wound up taking the equivalent of a major's worth of classes in biology and supporting sciences in addition to those in his major. Dwyer subsequently encouraged Jerry to apply to Tulane University, where eventually he was advised by the noted naturalist and botanical historian, Joseph Ewan while supported by a predoctoral fellowship from NASA. It was during his work at Tulane that Jerry

became interested in ferns, which would be the focus of his doctoral work and future career. Ewan and Walter Hodge (then at NSF) were among those who encouraged Jerry to accept a Master's Degree (in 1966) from Tulane and to apply to the doctoral program at Harvard University (although this meant abandoning his NASA fellowship for support through a grant from NSF). There, he completed his Ph.D. in 1971 under Rolla Tryon, one of the preeminent classical fern systematists of his time.

Jerry's doctoral work on the taxonomy of the tree fern genus *Nephelea* (Gastony, 1973) not only prepared him for a career in systematics, but it also stimulated his interest in related topics, such as the comparative morphology of fern spores, variation in the fern life cycle, and speciation. Jerry accepted a faculty position at Indiana University in 1970, straight from graduate school. His initial research in Bloomington focused primarily on the spore morphology of tree ferns (Gastony, 1974, 1979, 1981, 1982; Gastony and Tryon, 1976).

However, several years into his position, Jerry became aware that in order to lead a successful career in a department that emphasized evolutionary studies beyond the organismal level, he would have to expand the focus of his research to address basic questions in evolutionary biology. In order to gain technical skills that would allow him to broaden his research program. Jerry sat in on several courses at Indiana University on biochemistry and genetics. He then applied this knowledge to a new effort to adapt the developing field of isozyme electrophoresis to ferns. He also spent his first sabbatical in Leslie Gottlieb's lab at the University of California at Davis, where he perfected his isozyme techniques and began to apply them to evolutionary and population genetic studies in ferns. At the time, existing protocols to extract, resolve, and genetically interpret the banding patterns of common enzyme systems mostly did not work with ferns (Soltis *et al.*, 1983), and Jerry was challenged to prove himself in the Gottlieb lab. Ferns in the genus *Pellaea* are abundant and cytologically diverse in California, and these became Jerry's model system for many future studies involving taxonomic relationships, population genetics, formation of polyploids, and the contributions of apogamous taxa to fern evolution (Gastony and Gottlieb, 1982, 1985; Gastony, 1988, 1990, 1991, Gastony and Windham, 1989).

The coupling of classical and molecular techniques led to Jerry's pioneering work on fern isozymes, and his lab (known as "Sky Lab" because of its location on the top floor of Jordan Hall) became a popular destination and invaluable resource for graduate and postdoctoral students interested in plant systematics and evolution. In the mid-1980s, Jerry and his students and collaborators further expanded the lab's repertoire to include restriction-site variation of DNA. Jerry's lab was one of the first to use variation in fern chloroplast DNA to understand historical relationships among fern species and genera (Yatskievych *et al.*, 1988, Stein *et al.*, 1989; Gastony *et al.*, 1992). A few years later, Jerry began studying DNA sequence data for phylogenetic analyses of ferns, which eventually led to the first comprehensive phylogeny for ferns (Hasebe *et al.*, 1995). Most recently, his lab generated the first genetic linkage map for a

FIG. 1. Gerald J. Gastony working in the greenhouse at Indiana University in 2008.

fern, which will provide an important and permanent resource for fern genetics (Nakazato *et al.*, 2006).

Because of the great diversity of Jerry's contributions to fern systematics and evolution, it is difficult to summarize all of them here. For example, his early work on spore morphology of the Cyatheaceae (Gastony, 1974, 1979; Gastony and Tryon, 1976) provided some of the initial evidence that the prevailing generic classification was unnatural. He was the first to count the chromosomes of the sporophyte-less taxon, *Vittaria appalachiana* Farrar & Mickel, which required adapting existing cytological protocols to the special demands of mitotic cells in gametophytic tissue (Gastony, 1977). He also demonstrated that ferns have diploid isozyme expression patterns despite their high chromosome numbers and that, contrary to prevailing wisdom at the time, homosporous ferns are highly heterozygous rather than homozygous (Gastony and Gottlieb, 1982, 1985). He later showed that fern genes can become silenced following genome doubling (Gastony, 1991). His work on cheilanthoid ferns provided the first robust phylogeny of that large and taxonomically difficult group (Gastony and Rollo, 1995, 1998), but he also has made substantial contributions to the understanding of other fern groups, in such families as Apleniaceae (Gastony, 1971; Gastony, 1986; Gastony and Johnson, 2001), Onocleaceae (Gastony and Ungerer, 1997), and other subfamilies of Pteridaceae (Gastony and Baroutsis, 1975; Baroutsis and Gastony, 1978; Gastony and Johnson, 2001; Nakazato and Gastony, 2003).

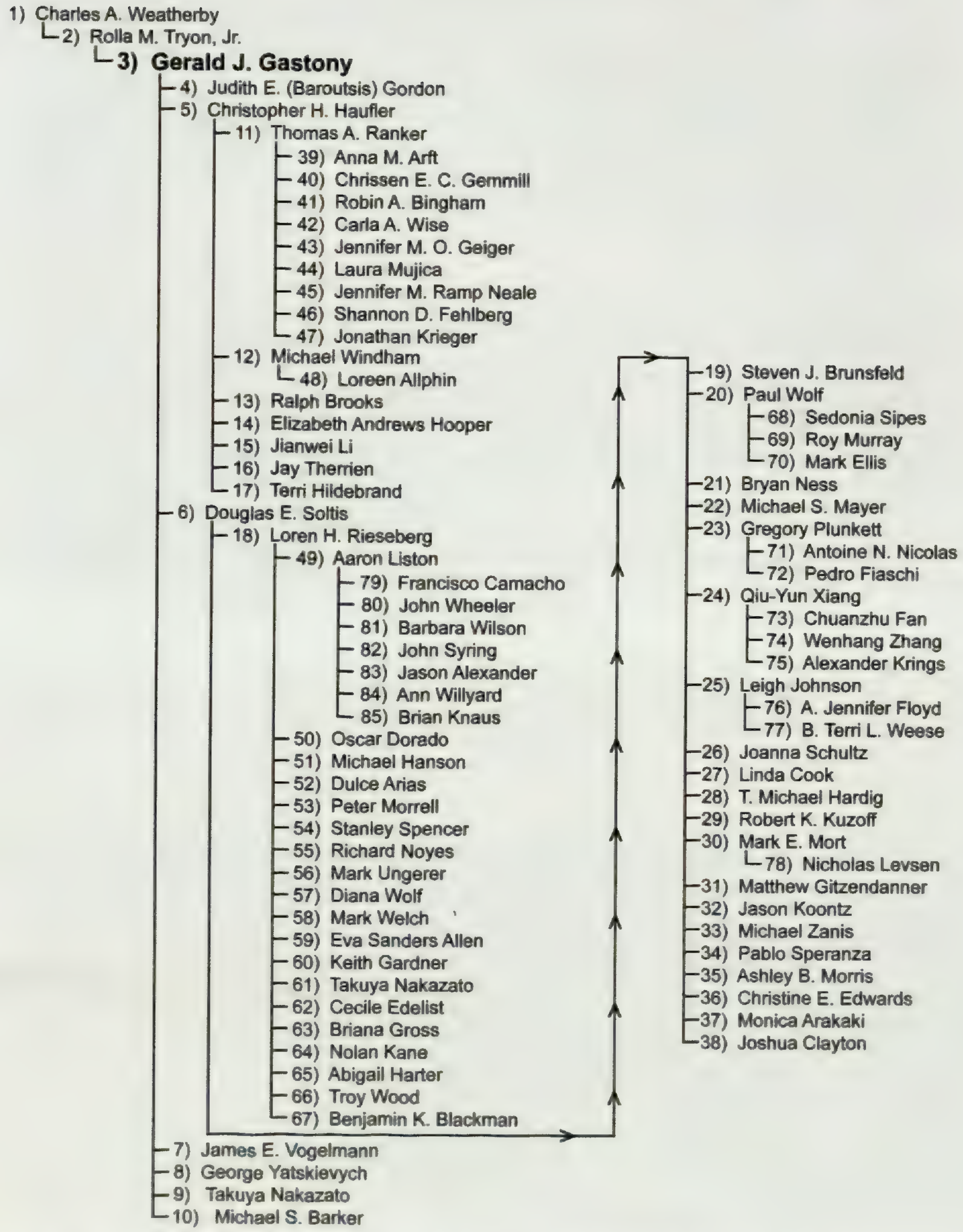

FIG. 2. An academic genealogy of Gerald J. Gastony, his botanical lineage, and his academic descendants. See Table 1 for further information on each person named.

In 1995, Jerry Gastony (Fig. 1) received the Edgar T. Wherry Award from the Botanical Society of America (Anonymous, 1995). In 2006, he was one of the honorees for a Centennial Medallion Award from the Botanical Society of America. He was chairman of the Pteridological Section of the Botanical

TABLE 1. Biographical summary of individuals in the Academic Genealogy of Gerald J. Gastony. See Fig. 2 for chronology and context.

1. Charles A. Weatherby. Academic grandfather. See *American Fern Journal* 40(1) for information.
2. Rolla M. Tryon, Jr. Academic father. Ph.D. Harvard University, 1941. See *American Fern Journal* 92(1): 1–9, 2002 for further information.
3. Gerald J. Gastony. Ph.D. 1971, Harvard University. Currently Professor of Biology Emeritus, Indiana University, Bloomington.
4. Judith E. (Baroutsis) Gordon. Ph.D. 1976 (as Judith G. Baroutsis), Indiana University, Bloomington. Currently Professor of Biology Emerita, Department of Biology, Augusta Sate University, Augusta, GA.
5. Christopher H. Haufler. Ph.D. 1977, Indiana University, Bloomington. Currently Professor and Chair, Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence.
6. Douglas E. Soltis. Ph.D. 1980, Indiana University, Bloomington. Currently Professor and Chair, Department of Botany, University of Florida, Gainesville.
7. James E. Vogelmann. Ph.D. 1983, Indiana University, Bloomington. Currently Research Ecologist, U.S. Geological Survey Earth Resources Observation and Science Center, Sioux Falls, SD and Adjunct Professor, South Dakota State University, Brookings.
8. George Yatskievych. Ph.D. 1990, Indiana University, Bloomington. Currently Curator and Director of the Flora of Missouri Project, Missouri Botanical Garden, St. Louis and Research Associate Professor and Adjunct Graduate Faculty, University of Missouri–St. Louis and Research Associate, Arizona-Sonora Desert Museum, Tucson.
9. Takuya Nakazato. Ph.D. 2005, Indiana University, Bloomington. Co-advised by Loren H. Rieseberg. Currently Assistant Professor, Department of Biology, University of Memphis, Memphis, Tennessee. Also see number 60,
10. Michael S. Barker. Ph.D. 2009, Indiana University, Bloomington. Co-advised by Loren H. Rieseberg. Currently Postdoctoral Associate, Department of Botany, University of British Columbia, Vancouver.
11. Thomas A. Ranker. Ph.D. 1987, University of Kansas. Currently Professor and Chair, Department of Botany, University of Hawaii at Manoa, Honolulu, HI.
12. Michael Windham. Ph.D. 1988, University of Kansas. Currently Research Scientist and Curator of Vascular Plants, Department of Biology, Duke University, Durham, NC.
13. Ralph Brooks. Ph.D. 1989, University of Kansas. Currently Senior Environmental Scientist Black & Veatch, Lake Oswego, OR.
14. Elizabeth Andrews Hooper. Ph.D. 1994, University of Kansas. Currently Associate Professor of Biology, Truman State University, Kirksville, MO.
15. Jianwei Li. Ph.D. 1996, University of Kansas. Currently Bioinformatics Engineer III, J. Craig Venter Institute, Rockville, MD.
16. Jay Therrien. Ph.D. 2003, University of Kansas. Currently Director of Sales, Asia Pacific and Japan, Illumina, Inc., Scoresby VIC, Australia.
17. Terri Hildebrand. Ph.D. 2005, University of Kansas. Currently Assistant Professor of Botany, Department of Biology, Southern Utah University, Cedar City, UT.
18. Loren H. Rieseberg. Ph.D. 1987, Washington State University. Currently Professor and Canada Research Chair, Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada and Distinguished Professor, Department of Biology, Indiana University, Bloomington.
19. Steven J. Brunsfeld. Ph.D. 1990, Washington State University. Professor, Department of Forest Resources, University of Idaho, Moscow. Deceased, 2007 (http://www.cnrhome.uidaho.edu/default.aspx?pid=96887).
20. Paul Wolf. Ph.D. 1990, Washington State University. Advised by Pamela Soltis, co-advised by Douglas Soltis. Currently Professor, Department of Biology, Utah State University, Logan.
21. Bryan Ness. Ph.D. 1992, Washington State University. Currently Associate Professor, Department of Biology, Pacific Union College, Angwin, CA.

TABLE 1. Continued.

22. Michael S. Mayer. Ph.D. 1993, Washington State University. Advised by Pamela Soltis, co-advised by Douglas Soltis. Currently Associate Professor, Department of Biology, University of San Diego, San Diego, CA.
23. Gregory Plunkett. Ph.D. 1994, Washington State University. Currently Curator and Director, Cullman Program in Molecular Systematic Studies, The New York Botanical Garden, Bronx, NY.
24. Qiu-Yun Xiang. Ph.D. 1995, Washington State University. Currently Associate Professor, Department of Plant Biology, North Carolina State University, Raleigh, NC.
25. Leigh Johnson. Ph.D. 1996, Washington State University. Currently Associate Professor and Herbarium Curator, Department of Biology, Brigham Young University, Provo, UT.
26. Joanna Schultz. Ph.D. 1996, Washington State University. Advised by Pamela Soltis, co-advised by Douglas Soltis. Currently Senior Consultant, Earth Informations Systems, Houston, TX.
27. Linda Cook. Ph.D. 1998, Washington State University. Advised by Pamela Soltis, co-advised by Douglas Soltis. Currently Lecturer part time, Washington State University, Pullman.
28. T. Michael Hardig. Ph.D. 1998, Washington State University. Advised by Pamela Soltis, co-advised by Douglas Soltis. Currently Associate Professor, Department of Biology, Chemistry, and Mathematics, University of Montevallo, Montevallo, AL.
29. Robert K. Kuzoff. Ph.D. 1998, Washington State University. Co-advised by Larry Hufford. Currently Associate Professor, Department of Biological Sciences, University of Wisconsin, Whitewater.
30. Mark E. Mort. Ph.D. 1999, Washington State University. Currently Associate Professor, Department of Ecology and Evolutionary Biology and Associate Curator of the McGregor Herbarium, University of Kansas, Lawrence.
31. Matthew Gitzendanner. Ph.D. 2000, Washington State University. Advised by Pamela Soltis, co-advised by Douglas Soltis. Currently Associate Scientist, Department of Botany, University of Florida, Gainesville.
32. Jason Koontz. Ph.D. 2000, Washington State University. Advised by Pamela Soltis, co-advised by Douglas Soltis. Currently Assistant Professor, Department of Biology, Augustana College, Rock Island, IL.
33. Michael Zanis. Ph.D. 2002, Washington State University. Currently Assistant Professor, Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN.
34. Pablo Speranza. Ph.D. 2005, University of Florida. Advised by Pamela Soltis, co-advised by Douglas Soltis. Currently Profesor Adjunto de Fitotecnia, Departamento de Biología Vegetal, Universidad de la República, Montevideo, Uruguay.
35. Ashley B. Morris. Ph.D. 2006, University of Florida. Advised by Pamela Soltis, co-advised by Douglas Soltis. Currently Assistant Professor, Department of Biology, University of South Alabama, Mobile.
36. Christine E. Edwards. Ph.D. 2007, University of Florida. Co-advised by Douglas Soltis and Pamela Soltis. Currently Postdoctoral Research Scientist, Department of Botany, University of Wyoming, Laramie.
37. Monica Arakaki. Ph.D. 2008, University of Florida. Currently Postdoctoral Fellow, Department of Ecology and Evolutionary Biology, Brown University, Providence, RI.
38. Joshua Clayton. Ph.D. 2008, University of Florida. Currently seeking employment in UK.
39. Anna M. Arft. Ph.D. 1995, University of Colorado, Boulder. Currently homemaker.
40. Chrissen E. C. Gemmill. Ph.D. 1996, University of Colorado, Boulder. Currently Senior Lecturer (≈ Associate Professor in U.S.), Department of Biological Sciences, University of Waikato, Aotearoa, New Zealand.
41. Robin A. Bingham. Ph.D. 1997, University of Colorado, Boulder. Currently Professor, Department of Natural and Environmental Sciences, Western State College, Gunnison, CO.

TABLE 1. Continued.

42. Carla A. Wise. Ph.D. 1997, University of Colorado, Boulder. Yan Linhart, co-advisor. Currently independent Environmental and Science Writer, Corvallis, OR.
43. Jennifer M. O. Geiger. Ph.D. 2003, University of Colorado, Boulder. Currently Associate Professor, Department of Natural Sciences, Carroll College, Helena, MO.
44. Laura Mujica. Ph.D. 2004, University of Colorado, Boulder (as Laura Mujica-Crapanzano). Patrick Bourgeron co-advisor. Currently Term Assistant Professor, Chemistry Department, University of Alaska, Anchorage, AK.
45. Jennifer M. Ramp Neale. Ph.D. 2005, University of Colorado, Boulder. Sharon Collinge, co-advisor. Currently Associate Director of Research and Director of the Conservation Genetics Program, Denver Botanic Gardens, Denver, CO.
46. Shannon D. Fehlberg. Ph.D. 2006, University of Colorado, Boulder. Currently Conservation Biologist, Desert Botanical Garden, Phoenix, AZ.
47. Jonathan Krieger. Ph.D. 2007, University of Colorado, Boulder. Robert P. Guralnick co advisor. Currently Postdoctoral Research Associate, Department of Palaeontology, The Natural History Museum, London.
48. Loreen Allphin. Ph.D. 1996, University of Utah, Salt Lake City. Advised by Delbert Wiens, co-advised by Michael Windham. Currently Associate Professor, Department of Plant and Wildlife Sciences, Brigham Young University, Provo, UT.
49. Aaron Liston. Ph.D. 1990, Claremont Graduate University, Claremont, CA. Nominally advised by Thomas S. Elias, co-advised by Loren H. Rieseberg. Currently Professor, Department of Botany and Plant Pathology and Director of the OSU Herbarium, Oregon State University, Corvallis, OR.
50. Oscar Dorado. Ph.D. 1992, Claremont Graduate University. Currently Professor, Universidad Autónoma del Estado de Morelos, Cuernavaca, Mexico.
51. Michael Hanson. Ph.D. 1993, Claremont Graduate University. Currently tenured botany Instructor, Bellevue College, Bellevue, WA.
52. Dulce M. Arias. Ph.D. 1994, Claremont Graduate University. Currently Professor, Universidad Autónoma del Estado de Morelos, Cuernavaca, Mexico.
53. Peter Morrell. Ph.D. 1997, Claremont Graduate University. Currently Senior Research Geneticist, Monsanto Co., St Louis, MO.
54. Stanley Spencer. Ph.D. 1997, Claremont Graduate University. Currently Senior Biologist at LSA Associates, Inc. [environmental consulting firm], Riverside, CA.
55. Richard Noyes. Ph.D. 1999, Indiana University, Bloomington. Currently Assistant Professor, Department of Biology, University of Central Arkansas, Conway, AR.
56. Mark Ungerer. Ph.D. 2000, Indiana University, Bloomington. Currently Assistant Professor, Division of Biology, Kansas State University, Manhattan, KS.
57. Diana Wolf. Ph.D. 2000, Indiana University, Bloomington. Currently Assistant Professor, Institute of Arctic Biology, University of Alaska, Fairbanks AK.
58. Mark Welch. Ph.D. 2002, Indiana University, Bloomington. Currently Assistant Professor, Department of Biological Sciences, Mississippi State University, Mississippi State, MS.
59. Eva Sanders Allen. Ph.D. 2002, Indiana University, Bloomington. Ellen Ketterson, co-advisor. Currently Grants Specialist, Department of Biology, Indiana University, Bloomington.
60. Keith Gardner. Ph.D. 2004, Indiana University, Bloomington. Currently Postdoctoral Fellow, Royal Botanic Garden, Edinburgh, UK.
61. Takuya Nakazato. Ph.D. 2005, Indiana University, Bloomington. Co-advised by Gerald J. Gastony. Currently Assistant Professor, Department of Biology, University of Memphis, Memphis, Tennessee. Also see number 9.
62. Cécile Edelist. Ph.D. 2007, Université Paris-Sud 11, Orsay, France. Advised by Christine Dillmann and Delphine Sicard, co-advised by Loren H. Rieseberg. Currently research engineer, Conservation des Espèces, Restauration et Suivi des Populations, National Museum of Natural History, Paris, France.

TABLE 1. Continued.

63. Briana Gross. Ph.D. 2007, Indiana University, Bloomington. Co-advised by Elizabeth Kellogg, University of Missouri, St. Louis. Currently Postdoctoral Research Fellow, Department of Biology, Washington University, St. Louis, MO.
64. Nolan Kane. Ph.D. 2007, Indiana University, Bloomington. Currently Postdoctoral Research Associate, Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada.
65. Abigail Harter. Ph.D. 2008, Indiana University, Bloomington. Currently Postdoctoral Fellow, University of Edinburgh, UK.
66. Troy Wood. Ph.D. April 2009, Indiana University, Bloomington.
67. Benjamin K. Blackman. Ph.D. May 2009, Indiana University, Bloomington. Co-advised by Scott Michaels.
68. Sedonia Sipes. Ph.D. 2001, Utah State University. Currently Associate Professor, Department of Plant Biology, Southern Illinois University, Carbondale, IL.
69. Roy Murray. Ph.D. 1997, Utah State University. Currently code hacker, IEM.
70. Mark W. Ellis. Ph.D. May, 2009, Utah State University, Logan, UT.
71. Antoine N. Nicolas, Ph.D. May 2009, Virginia Commonwealth University, Richmond, VA.
72. Pedro Fiaschi Ph.D. anticipated, August 2009, Virginia Commonwealth University, Richmond, VA.
73. Chuanzhu Fan. Ph.D. 2003, North Carolina State University. Currently Assistant Research Scientist, Arizona Genomics Institute, University of Arizona, Tucson, AZ
74. Wenhang Zhang. Ph.D. 2006, North Carolina State University. Michael Purugganan, co-advisor. Currently Postdoctoral Fellow, Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA.
75. Alexander Krings. Ph.D. 2007, North Carolina State University. Jon M. Stucky, co-advisor. Currently Extension Assistant Professor and Director of the Herbarium, Department of Plant Biology, North Carolina State University, Raleigh, NC.
76. A. Jennifer Floyd. Ph.D. 2000, North Carolina State University. Nina Allen, co-advisor. Most recently Assistant Professor, Biology Program, University of Guam, Mangilao, Guam.
77. B. Terri L. Weese. Ph.D. 2004, Brigham Young University. Currently Editor, Australian Plant Name Index (APNI), CSIRO Plant Industry, Canberra, Australia.
78. Nicholas Levsen. Ph.D. 2008, University of Kansas. Currently Postdoctoral Fellow, Institute of Arctic Biology, University of Alaska, Fairbanks, AK.
79. Francisco J. Camacho. Ph.D. 1999, Oregon State University. James M. Trappe co-advisor. Currently homemaker, San Juan Capistrano, CA.
80. John Wheeler. Ph.D. 1998, Oregon State University. Currently Associate Professor, Department of Biology, University of Wisconsin, River Falls
81. Barbara Wilson. Ph.D. 1999, Oregon State University. Currently Partner, *Carex* Working Group LLC [botanical consulting firm], Eugene, OR.
82. John Syring. Ph.D. 2006, Oregon State University. Co-advised by Richard C. Cronn, USDA Forest Service PNW. Currently Assistant Professor, Linfield College, McMinnville, OR.
83. Jason Alexander. Ph.D. 2007, Oregon State University. Currently Herbarium Curator, Utah Valley University, Orem, UT.
84. Ann Willyard. Ph.D. 2007, Oregon State University. Currently Post Doctoral Fellow, Department of Biology, University of South Dakota, Vermillion, SD. Assistant Professor, Department of Biology, Hendrix College, Conway, AR starting August, 2009.
85. Brian Knaus. Ph.D. 2008, Oregon State University. Co-advised by Richard C. Cronn, USDA Forest Service PNW. Currently Postdoctoral Research Geneticist, USDA Forest Service PNW, Corvallis, OR.

Society of America (1979–1980) and also served as vice president (1994–1996) and president (1996–1998) of the American Fern Society. He has been an Associate Editor of the *American Fern Journal* since 1973 and was editor-in-chief of *Systematic Botany* from 1992 through 1995. Thus far, three species of plants new to science have been named in his honor: a Caribbean moss, *Macrocoma gastonyi* Norris & Vitt (1973); a Mexican polystichoid fern *Phanerophlebia gastonyi* Yatskievch (1992), and the uncommon allopolyploid *Pellaea gastonyi* Windham (1993).

In addition to his contributions to scientific research and service to several scientific societies, Jerry Gastony has been a caring and skilled teacher of both undergraduate and graduate students. His Vascular Plants course was widely recognized as one of the best courses in the Department of Biology at Indiana University, and in 2001 he was honored with the Department of Biology Senior Class Award for Teaching Excellence in Biology and Dedication to Undergraduates. He has also been a much loved and respected mentor to a small dynasty of graduate students, several of whom have gone on to become eminent plant systematists in their own right (Fig. 2, Table 1). During his tenure as director of the Evolution, Ecology, and Behavior Graduate Program in the IU Department of Biology from 1991 to 2002, this program developed into one the strongest of its kind in the country. Even after Gerald Gastony's retirement in 2006, he has continued to be a major force in pteridology and to interact with many researchers and students in the field.—Michael S. Barker, Department of Botany, University of British Columbia, 3529-6270 University Blvd, Vancouver, BC V6T 1Z4, CANADA, and Department of Biology, Indiana University Jordan Hall 142, 1001 E Third St., Bloomington, IN 46405-3700 and George Yatskievych, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299

Gels and Genetics: The Historical Impact of Isozymes on Paradigm Shifts in Hypotheses about Fern Evolutionary Biology.—Although it is comforting when new discoveries confirm established hypotheses, it is positively exciting when novel techniques and observations demand rejection of reigning textbook concepts. The history of genetics for homosporous ferns is an exemplar of how technical innovations and discoveries lead to significant modifications of our working models in biology. Homosporous ferns were originally placed in the mysterious group called the "cryptogams" because, unlike their "phanerogamic" cousins, their manner of breeding was hidden from obvious observation and investigation. Once botanists began culturing the gametophytes of ferns, their reproductive biology was revealed, and a method for conducting genetic experiments (crosses and progeny rearing) became available. The earliest studies of fern genetics were those of Lang (1923) and Anderson-Kottö (1931), who demonstrated that most ferns showed simple Mendelian inheritance of traits. In 1950, Irene Manton published her magnum opus, ushering in a new era of genetic and biosystematic research on seed-free plants. Manton's extensive survey demonstrated that most ferns had

extraordinarily high chromosome numbers and that often what appeared to be polymorphic species were actually reticulate complexes of diploid species and their allopolyploid derivatives. This research helped to demonstrate the importance of including genetic aspects of species in understanding their origins and their population dynamics.

In the 1970s, Edward Klekowski (1979) brought a renewed focus to fern genetics by developing logically consistent and compelling correlations and hypotheses about the evolutionary biology of homosporous vascular plants. Klekowski observed that because homosporous ferns had potentially bisexual gametophytes they should be highly inbred, and because these plants have high chromosome numbers, they should be polyploid. Klekowski further hypothesized that this polyploidy could represent an adaptive response that would buffer the homozygotizing effects of consistent inbreeding. Genetic variation stored among the several to many homoeologous genomes contained in polyploids could be released by non-homologous pairing mistakes during meiosis. Indeed, Hickok (e.g., 1978) provided evidence consistent with pairing between homoeologs. Klekowski's hypotheses were intriguing because if accurate they provided a different genetic system and different evolutionary trajectory for homosporous vascular plants.

- Population variation would be reduced and polymorphism constrained.
- Polysomic inheritance would require different algorithms for estimating the population genetic dynamics of homosporous vascular plants.
- If single spores could germinate to become bisexual gametophytes that generated sporophyte offspring, wind dispersal and migration would surmount most geographic barriers and lead to large species ranges.

Although some breeding experiments and chromosomal studies proved to be consistent with Klekowski's hypotheses, central implications of them could not be addressed until enzyme electrophoresis provided a window on molecular genetics. Whereas the hypotheses predicted that ferns should have numerous duplicated genetic loci and be predominantly homozygous, isozymes demonstrated that species with generically basal chromosome numbers were genetically diploid and possessed numerous heterozygous loci (Gastony and Gottlieb, 1982; Haufler and Soltis, 1986). These discoveries required revised hypotheses and forced a revolution in modeling population-level phenomena for ferns.

- Mechanisms promoting outcrossing were explored and verified through coordination of laboratory and field studies (e.g., Haufler and Soltis, 1984).
- Given a new (higher base numbers) starting point, polyploidy levels in homosporous vascular plants actually approximated those of other plant groups (Vida, 1976).
- No longer constrained by lethargic rates of change because of polygenic systems, it was reasonable to posit that diploid ferns could adapt and diversify along with their seed plant descendants (Schneider *et al.*, 2004).
- At the species level, migration via single spores became a specialized rather than a standard capacity for ferns (Haufler, 2002).
- Fern biogeographers were required to consider a new variety of possible outcomes from dispersal and vicariance (e.g., Wolf *et al.*, 2001).

- Within populations, standard diploid-based models of population genetics obtain. Most diploids have random-mating breeding systems with inbreeding restricted to specialist species, those with subterranean gametophytes, and (of course) polyploids (Ranker and Geiger, 2008).

Dismissing ferns as stagnant evolutionary dead-ends ceased to be an option, and with exciting new evidence from DNA and genomic studies, new vistas are opening all the time.

Discovering the paradox that ferns had high chromosome numbers but were genetically diploid necessarily led researchers to ask how this unusual condition could have evolved. One hypothesis was that ferns differed (once again) from other organisms and the lineage started with a larger number of chromosomes (Soltis and Soltis, 1987). A second hypothesis stated that ferns (and other homosporous vascular plants) accumulated chromosomes through cycles of polyploidy events, followed by a return to genetic diploidy through gene silencing (Haufler, 1987). Why ferns retain chromosomes after silencing half their genes remains unclear (and does suggest they differ from other organisms), although it may be related to strong genetic control of bivalent formation (multivalents–that can result in chromosome losses–are rare in ferns having a balanced number of chromosome sets). Experiments and observations aimed at testing these hypotheses (Pichersky *et al.*, 1990; Gastony, 1991; McGrath and Hickok, 1999; Nakazato *et al.*, 2008) have all demonstrated that ferns having chromosome numbers that are basic within genera appear to have experienced ancient polyploidy followed by gene silencing. Support for the polyploidy plus silencing hypothesis is also consistent with new evidence that plant genomes are remarkably volatile and fluid (e.g., Adams and Wendel, 2005).

Resolving these genetic mysteries of vascular cryptogams leads to a whole new set of open questions:

- We know little or nothing about the actual processes involved with gene silencing in ferns. What is the mechanism that results in the paradoxical genetic constitution of the homosporous vascular plants?
- The majority of studies on fern genetics have focused on temperate groups. With most diversity in the tropics, and the origin of temperate groups tied to tropical ancestors, we need to know more about how tropical populations work and whether the conclusions drawn from studies of temperate populations apply to tropical ones.
- We still know surprisingly little about the actual mechanisms that control breeding systems in ferns. More studies that coordinate laboratory analyses of gametophyte biology with surveys of natural populations may help to link mechanisms with observed patterns of genetic variation.
- Although assumptions about trends in species migration have been proposed, the processes that actually result in the founding of new populations remains mysterious. What happens when spores arrive in a new location? What limitations are imposed on species migration by outcrossing breeding systems? Again, coordination of lab and field studies may help to resolve these open questions.
- Perhaps the biggest remaining mystery involves the origin of new species. With demonstrations that fern speciation takes advantage of new habitats opened when angiosperms diversify, it may be possible to study early stages in the cladogenetic process of ferns.

These and other vistas await future generations of scientists interested in understanding the fascinating world of homosporous vascular plants and revealing the cryptic nature of their biology and genetics.—CHRISTOPHER H.

HAUFLER, Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence, KS 66045.

Using Plastid and Nuclear DNA Sequences to Redraw Generic Boundaries and Demystify Species Complexes in Cheilanthoid Ferns.—Cheilanthoid ferns constitute a monophyletic group of 400–500 species within the Pteridaceae (Smith *et al.*, 2006; Schuettpelz and Pryer, 2007; Schuettpelz *et al.*, 2007). They are noteworthy for their ability to colonize xeric and semi-xeric habitats, niches that are rarely exploited by other ferns (Tryon and Tryon, 1979, 1982). Relationships within this lineage are highly problematic, and cheilanthoids have been called "the most contentious group of ferns with respect to a practical and natural generic classification" (Tryon and Tryon, 1982: 248). It is not surprising, then, that molecular phylogenetic analyses to date have revealed that most of the larger cheilanthoid genera are polyphyletic (Gastony and Rollo, 1998; Kirkpatrick, 2007; Prado *et al.*, 2007; Schuettpelz *et al.*, 2007; Zhang *et al.*, 2007; Rothfels *et al.*, 2008). Cheilanthoid ferns have long been a topic of interest for Dr. Gerald Gastony, the honoree of this collection of papers. His contributions run the gamut from studies of chromosome numbers and apomixis in *Bommeria* E. Fourn. (Gastony and Haufler, 1976), through genetic analyses of various species groups (Gastony, 1988; Gastony *et al.*, 1992), to documenting tetrasomic inheritance and gene silencing in polyploids (Gastony, 1990, 1991), and maternal inheritance of plastids in *Pellaea* Link (Gastony and Yatskievych, 1992). His phylogenetic studies of cheilanthoids (Gastony and Rollo, 1995, 1998) were the first to demonstrate that *rbcL* sequences could provide a valuable, independent tool for circumscribing genera in this taxonomically controversial group of ferns.

We are now poised to take the "next step" toward redefining generic boundaries among the cheilanthoids. It is clear that the number of genes and taxa analyzed must be significantly increased if we hope to obtain a robust phylogeny of the group. To this end, we have initiated a large-scale phylogenetic study using DNA sequences derived from three plastid regions (*rbcL*, *atpA*, *trnG-R*). To date, we have sequenced all three plastid regions (representing nearly 4000 base pairs) for 157 species. Maximum likelihood analyses of these data identify seven, well-supported subclades of cheilanthoid ferns (Fig. 3).

Ludens clade.—Previously published analyses (Schuettpelz *et al.*, 2007; Zhang *et al.*, 2007) revealed that *Doryopteris ludens* (Wall. ex Hook.) J. Sm. is not closely related to most taxa traditionally placed in this genus, including the type species, *D. palmata* (Willd.) J. Sm. Whereas *Doryopteris* J. Sm. in the strict sense is strongly supported as a member of the hemionitid clade (Fig. 3), *D. ludens* and its close allies appear to represent a rather isolated lineage within the Pteridaceae. Analyses by Schuettpelz *et al.* (2007) resolved *D. ludens* as sister to all other cheilanthoid ferns while those of Zhang *et al.* (2007) suggested a possible affinity to other pteroid lineages. Though the placement of this species varies depending on taxon sampling, it is clear that it

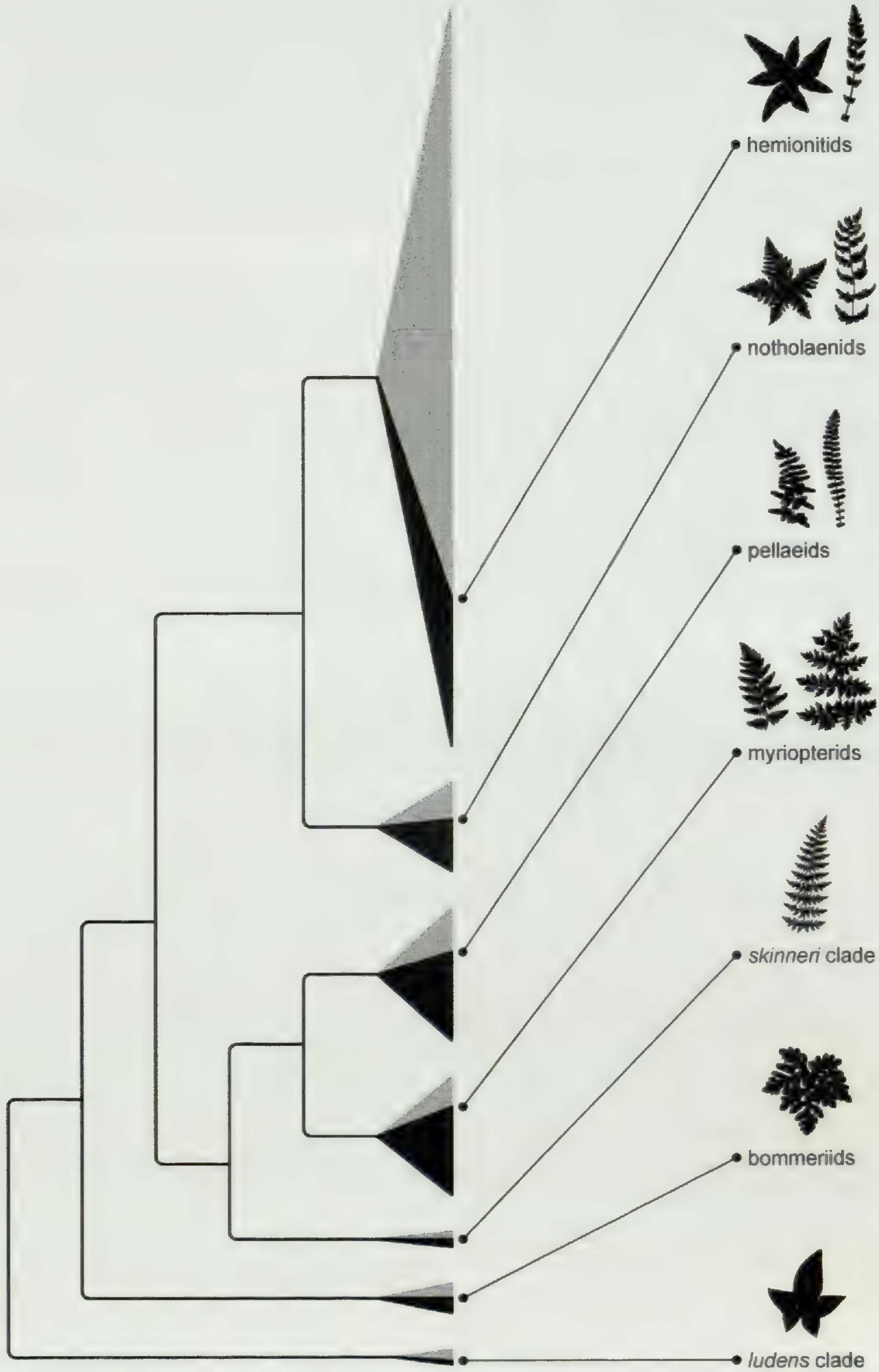

FIG. 3. Summary of phylogenetic relationships within cheilanthoid ferns. Topology results from maximum likelihood analyses of *atpA*, *rbcL*, and *trnG-R* sequence data for 157 species; tree rooted with *Doryopteris ludens*. Thumbnails identify seven, well-supported cheilanthoid clades. Triangles indicate proportion of named species belonging to each clade; darker portion of each triangle represents the proportion of species included in the current analysis.

is more closely related to cheilanthoid ferns than to any other potential outgroup sampled to date. For this reason, we have used it in our analyses to root the remaining cheilanthoid tree. The *D. ludens* clade encompasses a total of four species (only one of which is included in our sample) whose combined range extends from continental Asia to New Guinea. Because its phylogenetic divergence and geographic isolation from *Doryopteris s.s.* are substantial, this lineage is in the process of being transferred to a new genus: "*Calciphilopteris*" (Yesilyurt and Schneider, in press).

Bommeriids.—As shown in earlier studies (Gastony and Rollo, 1995, 1998), species of *Bommeria sensu lato* (including *B. elegans* (Davenp.) Ranker & Haufler; see Ranker and Haufler, 1990) are sister to all cheilanthoids other than the *D. ludens* clade. Our data confirm Ray Cranfill's (unpubl. data) assignment of *Cheilanthes brandegeei* D. C. Eaton to this clade, suggesting that the circumscription of *Bommeria* may need to be expanded yet again. Some species that Tryon and Tryon (1982) considered close relatives of *C. brandegeei* are strongly supported as members of the notholaenid clade in our analyses (Rothfels *et al.*, 2008), and these already have been transferred to *Notholaena* (Yatskievych and Arbelaez, 2008). The remaining members of the "*C. brandegeei* group" (*sensu* Tryon and Tryon, 1982) need to be sampled before the bommeriid clade can be accurately delimited. Based on available data, we estimate that this lineage ultimately will encompass about 2% of cheilanthoid species, half of which have now been included in our analyses.

Skinneri clade.—In a recent parsimony analysis of *rps4*, *rps4-trnS*, and *trnL-F* sequences by Kirkpatrick (2007), *Cheilanthes skinneri* (Hook.) R.M. Tryon & A.F. Tryon was weakly supported as sister to all cheilanthoids other than *Bommeria* (the *ludens* clade was not included in her sampling). In our studies, this taxon is strongly supported as sister to the myriopterid + pellaeid clade; together, these three clades are sister to the notholaenid + hemionitid clade (Fig. 3). Our molecular data also support a close relationship between *C. skinneri* and *C. lozanoi* (Maxon) R.M. Tryon & A.F. Tryon, an association previously proposed based on morphology (Mickel, 1987). Although these species have been transferred back and forth between *Pellaea* (in the pellaeid clade) and *Cheilanthes* (hemionitid clade) in the past, our data indicate that neither generic placement is tenable. Mickel (1987) identified several other taxa that may be related to *C. skinneri*, and these must be sampled before we can adequately circumscribe the clade and determine the correct generic name for it. Based on the available data, we estimate that this primarily North American lineage will include 4–5 species (about 1% of cheilanthoid diversity), two of which were included in the current analysis.

Myriopterids.—This clade encompasses a group of primarily North American species traditionally placed in *Cheilanthes*. A similar assemblage, also sister to the pellaeid clade, was recovered by both Gastony and Rollo (1998) and Kirkpatrick (2007). Our analyses indicate that this group is only distantly related to the type species of *Cheilanthes* (*C. micropteris* Sw., a member of the hemionitid clade) and, as such, all included taxa will need to be transferred to another genus (Grusz *et al.*, in prep.). The type species of *Myriopteris* Fée

(1852), *Cheilosoria* Trev. (1877), and *Pomatophytum* M.E. Jones (1930) all belong to this clade, so there is no shortage of potential names. The challenge will be to identify morphological features that consistently separate this group from *Cheilanthes sensu stricto*. We estimate that this lineage comprises approximately 10% of cheilanthoid diversity; 75% of recognized species have been sampled to date.

Pellaeids.—In addition to *Pellaea s.s.* (described by Link in 1841), this clade includes four genera named within the last 70 years: *Argyrochosma* (J. Sm.) Windham, *Astrolepis* D.M. Benham & Windham, *Paraceterach* Copel., and *Paragymnopteris* K.H. Shing. As revealed by earlier molecular analyses (Gastony and Rollo, 1998; Kirkpatrick, 2007), *Argyrochosma* (with ca. 30 species) is sister to all other pellaeids and can continue to be recognized as a distinct genus as proposed by Windham (1987). The other three genera, although morphologically more divergent than *Argyrochosma*, are nested within the traditional circumscription of *Pellaea* section *Pellaea*. It appears that members of this clade have switched from a typical *Pellaea* morphology (highly divided, nearly glabrous leaves) to an *Astrolepis-Paraceterach-Paragymnopteris* morphology (usually simply pinnate, densely scaly or hairy leaves) on no less than three occasions on three different continents. The taxonomic problems posed by this situation are not easily resolved; Kirkpatrick (2007) provided a good discussion of the potential synapomorphies of each pellaeid subclade and the various nomenclatural options. The pellaeid clade comprises about 12% of cheilanthoid diversity; 65% of the species are represented in our analyses and additional representatives were sampled by Kirkpatrick (2007).

Notholaenids.—This primarily North American lineage, the subject of a recent study by Rothfels *et al.* (2008), is sister to the large, cosmopolitan hemionitid clade. Most of the species included in the notholaenids are farinose, with abaxial leaf surfaces covered by "powdery" (predominantly flavonoid) deposits produced by underlying glandular trichomes. This feature has often been considered a synapomorphy for the genus *Notholaena* R. Br. (*sensu* Yatskievych and Smith, 2003), but our data place two nonfarinose taxa deep within the clade and a strongly glandular, but non-farinose, species as the earliest diverging branch. Additional morphological studies are underway (Rothfels *et al.*, in prep.) to identify characters that can be used to circumscribe an expanded *Notholaena*. This lineage comprises roughly 8% of cheilanthoid diversity; 60% of recognized species have been sampled to date.

Hemionitids.—This is, by far, the largest and most diverse clade of cheilanthoids; its members are found on every continent except Antarctica and the geographic ranges of two species, *Cheilanthes farinosa* (Forssk.) Kaulf. and *C. concolor* (Langsd. & Fisch.) R.M. Tryon & A.F. Tryon, cover most of the subtropics (Tryon and Tryon, 1973). The lineage includes the type species of more than a dozen genera named between 1753 (*Hemionitis* L.) and 1991 (*Pentagramma* Yatsk., Windham & E. Wollenw.). Nearly all of these generic names are associated with well-supported subclades in our analyses, but relationships among these groups are largely unresolved in the plastid tree.

The hemionitid lineage appears to have undergone a rapid radiation (possibly associated with its colonization of new habitats and continents), and much additional data will be needed to clarify generic boundaries in this group. We estimate that this lineage comprises about 67% of cheilanthoid diversity; only 20% of known species are represented in the current analysis.

Future directions.—Ultimately, we hope to include more than 60% of cheilanthoid species in our studies, with a special emphasis on under-sampled diversity hotspots in South America and Africa. The type species of all validly named genera will be sampled, as well as the majority of species of uncertain or disputed relationship. Phylogenetic analyses of these plastid DNA sequences will be used to identify well-supported monophyletic lineages. These clades can then be evaluated for morphological synapomorphies that will provide the foundation for a revised generic classification.—Michael D. Windham, Layne Huiet, Eric Schuettpelz, Amanda L. Grusz, Carl Rothfels, and James Beck, Department of Biology, Duke University, Durham, NC 27708-0339, George Yatskievych, Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299, and Kathleen M. Pryer, Department of Biology, Duke University, Durham, NC 27708-0339.

Phylogenetic Use of Inversions in Fern Chloroplast Genomes.—Evolutionary studies at the genome level are nothing new, even in ferns, for which the earliest approaches can be attributed to the cytogenetic investigations of Irene Manton (Manton, 1950). Yet, within a decade of the development of recombinant DNA techniques, researchers were examining genomes through the study of DNA rather than chromosomes. This began with the pioneering work of Jeffrey Palmer and Diana Stein who demonstrated the utility of variation in the chloroplast genome for evolutionary studies in land plants, including ferns (Palmer, 1987; Palmer and Stein, 1982; Stein *et al.*, 1986). Although the chloroplast genome (hereafter plastome) is generally conserved in structure (Palmer and Stein, 1986), it contains sufficient variation to be used at a wide range of phylogenetic scales.

Two general approaches were used to study structural variation in plastomes, both involving restriction site analysis. The first entailed mapping via heterologous probes. This provided data on structural changes which can be informative especially at deep phylogenetic levels (Raubeson and Jansen, 1992). Hasebe (1992) compared the plastome structure of the fern *Adiantum capillus-veneris* to that of tobacco and found that the gene order in *Adiantum* was reversed throughout much of the inverted repeat region. A series of inversions was necessary to explain the difference. Later Stein *et al.* (1992) attempted to examine this aspect of plastome structure across ferns. The study found that *Osmunda* has the tobacco gene order, whereas the remaining taxa studied (a tree fern and several polypods) all had the *Adiantum* gene order, with no additional changes in structure detected. The second approach to comparing plastomes used variation at the sequence level, detected by presence or absence of restriction sites. This approach was used for more

phylogenetically focused studies including polystichoid ferns (Stein *et al.*, 1989), Cyatheaceae (Conant *et al.*, 1994), and the genus *Pellaea* (Gastony *et al.*, 1992). Furthermore, maternal inheritance of the plastome was demonstrated in ferns (Gastony and Yatskievych, 1992).

By the 1990s, DNA sequencing had become feasible for systematists, such that it replaced restriction site analysis as the method of choice. This had several effects. One was that now researchers were more focused on variation in one or a few genes, those for which PCR and sequencing primers were first developed. However, the genome scale approach had been lost. Yet nucleotide variation was so useful that much of the overall framework of fern phylogeny was established (Hasebe *et al.*, 1994, 1995) using the gene *rbcL*, alone at first, but later adding data from additional genes (Pryer *et al.*, 2004).

We posit that evolutionary studies are now moving back to a genome scale perspective. This latest shift is again driven by technological advances, mostly those associated with high-throughput genomics, and the concomitant reduction in cost. Several researchers are starting to examine the highly complex nuclear genomes of ferns, and some of that work was included in this symposium. Our research group is focused on the plastome, of which two complete sequences are available for ferns: *Adiantum* (Wolf *et al.*, 2003) and *Angiopteris* (Roper *et al.*, 2007). Complete genome sequences provide advantages over the earlier mapping approaches: it is much easier to add taxa to a study and there is no need for additional cross probing. Also, the data provide both nucleotide data and genome structure data, deduced from gene order in the genome annotation. Although we do not yet have additional complete fern plastome sequences, we can use the information from *Angiopteris* and *Adiantum* to focus on a few key areas of the plastome. Now that a more robust phylogenetic framework is available for ferns, we can screen appropriate taxa to examine genome reorganization in more detail.

Our research asks two main questions: how phylogenetically informative is gene order, and what are the evolutionary dynamics of genome structure? Gene order can be phylogenetically informative if the individual events that make up a genome reorganization each fall on a different branch of the tree. Alternatively, if there are temporal destabilization events, then a series of rearrangements can occur on the same branch, reducing the number of informative characters, and in some cases preventing the interpretation of actual events (but still providing strong support for one branch). Furthermore, if physical hotspots for rearrangements are common then it is possible that characters of genome structure might be susceptible to homoplasy.

We used the plastome sequences of *Adiantum* and *Angiopteris* to design primers and used PCR and DNA sequencing to determine gene order in representatives of all major lineages of ferns. Here we focus only on a few regions that we know to vary, based on the two complete plastome sequences available. Details of the technique will be published elsewhere. We found that the complex reorganization of the inverted repeat in ferns (Stein *et al.*, 1992) occurred via two main events. *Angiopteris*, *Osmunda*, filmy ferns, and gleichenioid ferns all possess the "tobacco" (ancestral) gene order. The

schizaeoid ferns appear to have undergone one large (approximately 18 kb) inversion. The remaining lineages have a second large inversion which occurred after the first, and the result is the *Adiantum* gene order, with the rRNA genes occurring in the reverse order, as seen in all other land plant lineages studied to date. Thus, this structural reorganization appears to be comprised of two separate events that map consistently onto the fern phylogenetic framework of Pryer *et al.* (2004). However, other smaller rearrangements are composed of several inversions on the same branch, reducing their phylogenetic utility.

Despite major strides in our understanding of fern phylogeny, several key branches remain poorly resolved. One clade that seems to be well-supported is the monilophytes, which include the Ophioglossales/Psilotales, leptosporangiate ferns, marattioid ferns, and the horsetails (Pryer *et al.*, 2001), and we have found a 3 kb inversion that unites this clade. However, resolution among the four constituent lineages remains unclear. Another problematic area is the filmy ferns and gleichenioid ferns, which may be sister taxa, although the support for this is weak (Pryer *et al.*, 2004). As more ferns plastomes are sequenced it should be possible to discover more phylogenetically informative rearrangements that may help address such unresolved issues. Moreover, genome scale data can be used for more than just phylogenetic studies. For example, several plastomes contain nucleotide repeats that may be variable at the population level. Although shifts in the type of data collected may have been driven by advances in techniques, the trend seems to be an increased ability to generate large amounts of data. Thus, future developments will likely depend on the ability to manage and analyze large data sets.—Paul G. Wolf, Aaron M. Duffy, and Jessie M. Roper, Department of Biology, Utah State University, Logan, UT 84322-5305.

Fern Genome Structure and Evolution.—We now know that genome structure is a dynamic entity, and understanding how it evolves is of fundamental importance in biology. Ferns and seed plants are sister groups, and yet they show interesting differences in their genome structure. Hence, comparative analyses of their genome structure provide insights into what is unique in each group and how the genome structure differences evolve.

One major difference between the fern and seed plant genome is their chromosome numbers. Chromosome numbers of ferns, particularly homosporous ferns, are much higher than those of seed plants (Klekowski and Baker 1966), and the underlying cause of this phenomenon has long been of a great interest to biologists. It is traditionally thought that ferns have high chromosome numbers because they are polyploids (Wagner and Wagner, 1980; Grant, 1981). However, Gastony and Gottlieb (1982) showed that, despite their high chromosome numbers, ferns with the lowest chromosome numbers in their genus show isozyme expression patterns typical of diploid organisms.

To resolve the paradox of high chromosome numbers and diploid gene expression in ferns, Haufler (1987) hypothesized that they have acquired their

high chromosome numbers through repeated cycles of polyploidization and genome diploidization via gene silencing. Consistent with the Haufler's hypothesis, Gastony (1991) showed that duplicated genes in a recent tetraploid species have been progressively silenced since the polyploidization event. More recently, Nakazato *et al.* (2006) looked for evidence of past polyploidization event(s) in a 'diploid' fern at the DNA level, by constructing a linkage map of *Ceratopteris richardii* Brongn.. They detected a large number of duplicated genes, one of the highest proportions among past mapping studies in plants, supporting the hypothesis that ferns are polyploids. The distribution of gene duplicates in the genome, however, revealed no apparent homoeologous chromosomes, evidenced by clustering of sets of gene duplicates in different chromosomes. Nonetheless, statistical tests for clustering of gene duplicates at the genome level were highly significant, suggesting that *C. richardii* has a polyploid-like genome structure. Therefore, it appears that *C. richardii* and perhaps other 'diploid' ferns have experienced ancient polyploidization(s), but homeologous chromosomes have been broken up by subsequent gradual chromosomal rearrangements.

Furthermore, mapping the distribution of chromosome numbers on the known fern phylogeny revealed an apparent increase in the base chromosome numbers at the divergence between the water fern lineage and its sister, ca. 200 MYA (Nakazato *et al.*, unpubl.), although many exceptions to the pattern make it premature to draw a firm conclusion. Together with the results from the linkage mapping study (Nakazato *et al.*, 2006) and EST sequence analyses (Barker *et al.*, unpubl.), it can be concluded that ferns probably have experienced ancient polyploidization event(s).

Therefore, results from the past studies have largely support the Haufler hypothesis of repeated cycles of polyploidization and diploidization, and this phenomenon seems to explain the high chromosome numbers in ferns. However, it has become increasingly clear that polyploidization events are ubiquitous not only among ferns, but also among angiosperms (reviewed in Lockton and Gaut, 2005). Therefore, polyploidization events in ferns alone do not seem to explain the higher chromosome numbers in ferns than in seed plants, unless ferns experience more polyploidizations and extinctions of diploids.

Interestingly, the modes of chromosome structural evolution seem to be substantially different between ferns and seed plants, and this may help us to understand why ferns have higher chromosome numbers. Genome size and chromosome number are significantly positively correlated in ferns (Nakazato *et al.*, 2008), which is expected if no significant structural changes occur to chromosomes. However, no such correlation exists in angiosperms or gymnosperms, suggesting that chromosomal structure is highly dynamic in seed plants, but not in ferns. Also, the distributions of genome size and chromosome number are highly skewed toward low values in angiosperms, so there appears to be selection for small genome size and low chromosome number, but not among ferns.

So why are seed plant genomes more dynamic than fern genomes? Although we do not have good answers yet, we can speculate several alternatives. First, because most ferns are homosporous, and seed plants are heterosporous, this difference in reproductive system may induce selection on genome size and chromosome numbers, although the exact nature of this selection is not known. In support of this hypothesis, heterosporous ferns generally have low base chromosome numbers. Alternatively, chromosomal inheritance patterns may be fundamentally different between ferns and seed plants. Although multivalent formation is common among seed plants, in ferns multivalents that may start to form early in meiosis rarely survive to the late prophase stage. Finally, it is possible that ferns and seed plants have some differences in their genome composition. Transposable elements, in particular, are known to have a substantial contribution to genome size, especially in grasses (Bennetzen, 2002). Although highly speculative, it is possible that fern chromosome structure is highly stable because transposable element activity is lower relative to seed plants.

Answers to the question of why ferns and seed plants have different genome structure will come only from detailed empirical studies. It is highly desirable in future studies to investigate what makes up the large fern genomes and how they are different from those of seed plants. Also, we need to conduct hypothesis-driven studies to establish causal links between the genome structure differences and biological differences between ferns and seed plants, such as reproductive systems and chromosomal inheritance.—TAKUYA NAKAZATO, Dept. of Biology, The University of Memphis, 3700 Walker Ave., Memphis, TN 38152

Evolutionary Genomic Analyses of Ferns Reveal that High Chromosome Numbers are a Product of High Retention and Fewer Rounds of Polyploidy Relative to Angiosperms.—Ever since the first chromosome counts of homosporous pteridophytes revealed that they possess astonishingly high numbers of chromosomes, botanists have recognized the unique genomic composition of these plants. Basal chromosome counts for fern genera are significantly higher than similar values from angiosperms (homosporous ferns $n = 57.05$, angiosperms $n = 16$; Klekowski and Baker, 1966), a result that led early workers to assume that as many as 95% of ferns are polyploids. Numerous hypotheses have been proposed throughout the years to explain the origin and maintenance of these chromosome numbers, but Klekowski and Baker's (1966) hypothesis of homoeologous heterozygosity received the most attention as it was supported by early studies.

However, this hypothesis was refuted through a series of convincing isozyme investigations of fern genetics by Gastony and colleagues (Gastony and Gottlieb, 1982, 1985; Haufler and Soltis, 1986; Gastony, 1991). These studies demonstrated that homosporous fern species with the lowest numbers in their genera possess diploid gene expression patterns, and led to a

hypothesis that fern chromosome numbers are the product of numerous rounds of paleopolyploidy.

To test these hypotheses, I analyzed Sanger and 454-sequenced ESTs from four polypod fern species for evidence of ancient genome duplication. My analyses demonstrate that a single genome duplication occurred near the base of the polypod ferns, a lineage that comprises >80% of extant fern diversity. Combined with available fossil data, I also provide the first estimate of fern nuclear genome evolutionary rates with polypodiaceous nuclear genomes evolving at approximately 4.79×10^{-9} subst./syn. site/year and places the ancient genome duplication at 178 +/− 32 MYA.

Assuming that rates of chromosomal loss in ferns are comparable to angiosperms, this is fewer genome duplications than expected, as many angiosperms with much lower chromosome numbers have experienced numerous rounds of genome duplications (Cui *et al.*, 2006). To further elucidate this pattern, I calculated a rate of paleopolyploidzation for angiosperms and ferns from genomic data sets of 192 species (Barker *et al.*, in prep). This rate comparison reveals that, on average, ferns experience approximately half as many paleopolyploidizations as angiosperms.

So, why then do homosporous ferns possess so many more chromosomes than angiosperms? It appears that pteridophyte genomes are simply less dynamic than angiosperm genomes and maintain their chromosomes with higher fidelity. Consistent with this hypothesis of gene silencing with little loss of physical genetic material is the observation of significantly lower gene density in the *Ceratopteris* genome relative to seed plants (Rabinowicz *et al.*, 2005). Additionally, pteridophytes are the only lineage of vascular land plants that have a strong, positive correlation between genome size and chromosome number (Nakazato *et al.*, 2008). Possibly involved in the maintenance of these chromosomes is another peculiar pteridophyte trait, the strong bivalent pairing of chromosomes (Wagner and Wagner, 1980).

Further research is needed to identify the forces and mechanisms driving the striking differences in genome evolution and organization between seed plants and monilophytes. Perhaps the ultimate tool for addressing this question will be whole-genome sequences of homosporous and heterosporous ferns. Considering innovations in sequencing technology and the declining cost of sequencing, we are likely only a few years away from having such data and further elucidating this most outstanding pteridological mystery.—Michael S. Barker, Department of Botany, University of British Columbia, 3529-6270 University Blvd, Vancouver, BC V6T 1Z4, CANADA, and Department of Biology, Indiana University Jordan Hall 142, 1001 E Third St., Bloomington, IN 46405-3700.

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Review

Flora de Nicaragua. Tomo 4. Helechos, the flora edited by W. D. Stevens, O. M. Montiel, and A. Pool, the volume authored by L. D. Gómez and A. L. Arbeláez. Monographs in Systematic Botany 116: 1–348, + i–xvii. 2009. ISBN 0161-1542. Published by the Missouri Botanical Garden, St. Louis. Hard cover. Price: $109.00. ISBN 978-1-930723-87-0, 151 figures (full page plates), illustrations by A. L. Arbeláez. In Spanish. orders@mbgpress.info; web: http://mbgpress.info

This fourth and concluding volume of the Flora of Nicaragua (previous volumes, all seed plants, published in 2001, ISBN 9780915279951) contains coverage of the ferns and so-called "fern allies", and treats, in alphabetical order, 102 genera and 551 species known from the country. Twelve additional genera and 82 species are also given full treatment, and included in the keys, in expectation that many of these will eventually be found in Nicaragua, since the known distribution is in countries immediately to the north and/or south of Nicaragua. For each species, we are given the accepted name, citation of publication, basionym, salient synonyms, description, habitat, representative specimens (collector and number), range, occasional brief taxonomic discussion, an endangerment code, original line drawings (habit or diagnostic details), and a dot distribution map. Keys to species, but not to families or genera, are included. Introductory sections include a discussion and maps for concentration of both pteridophyte and vascular plant diversity in Nicaragua, and for density of collections within the country (by Stevens), discussion of conservation issues (by Montiel), placement of genera within families, and a general bibliography.

This volume presents an updated and more focused version, for Nicaragua only, of the earlier general flora for the region, *Flora Mesoamericana*, Vol. 1 (Davidse *et al.*, eds., 1995). There are, indeed, many first literature reports of species for Nicaragua, contained within this new work. The authors have generally adopted the most recent classification/taxonomy available for a given genus, with only minor exceptions: filmy ferns are presented in the traditional two genera system, rather than the recently published 9-genus classification by Ebihara *et al.* (2006); and *Cnemidaria* is treated apart from *Cyathea*. I noticed only a few questionable taxonomic decisions, e.g., the Committee for Pteridophyta has declared that the earliest typification of *Acrostichum ebeneum* L., by Tryon, must stand (Taxon 54:831. 2005), the effect being that that name is regarded as a synonym of *Pityrogramma tartarea* (Cav.) Maxon); *Pityrogramma ebenea* (L.) Proctor was used for this species by the authors. *Dryopteris rossii* is included in the flora on the basis of *Gómez 6160*, but I think it likely that this specimen is either mislocalized or misidentified. *Nephrolepis cordifolia* is said to be naturalized in the Neotropics, but the type, from the Dominican Republic, is conserved (McNeill *et al.*, eds.,Vienna Code, 2006), and the species generally considered to be native to at least parts of the New

World. Also, *Nephrolepis multiflora* is listed as a synonym of *N. hirsutula*, even though Hovenkamp and Miyamoto (Blumea 50: 279–322) included the former as a synonym of *N. brownii* (Desv.) Hovenkamp & Miyam., a species also accepted in the present flora; most likely, specimens assigned to *N. hirsutula* by Gómez and Arbeláez are really *N. brownii*, and specimens determined as the former are misidentifications. One somewhat confusing aspect of this flora is that a substantial number of species (e.g., *Psilotum nudum*, *Botrychium schaffneri*, *B. virginianum*, *Ophioglossum crotalophoroides*, *Hymenophyllum pulchellum*, *H. trapezoidale*, *H. undulatum*, and *Asplenium salicifolium*, to name a few) listed by Gómez (1976; Brenesia 8:41–57) in his enumeration of ferns of Nicaragua are included in the present flora on the *expectation* of their possible occurrence in Nicaragua—this, despite the statement by Gómez (1976, p. 41) that the earlier list was compiled from ferns "conocidos hasta la fecha como resultado de una revisión de literatura y el examen de varios miles de ejemplares colectado por mí y depositado en el Herbario Nacional de Costa Rica y mi herbario personal." One would have preferred an unambiguous statement to the effect that the current authors were now unable to verify the existence of the species in question in Nicaragua. This underscores the inadvisability of accepting range statements for floras on the basis of literature citations. I myself have been guilty of this (Smith, 1981, Flora of Chiapas), accepting, uncritically, range statements for species said to be in Nicaragua by Gómez (1976); in turn, my range statements (for Nicaragua) were taken up in the *Flora Mesoamericana* (Moran & Riba, 1995). In this way, the cycle of misinformation continues.

The largest pteridophyte genera for Nicaragua are *Thelypteris* s.l. (51 spp.), *Asplenium* (39 spp.), *Elaphoglossum* (28 spp.); *Trichomanes* s.l. (28 spp.), *Adiantum* (26 spp.), *Diplazium* (23 spp.), and *Selaginella* (21 spp). In fact, the 10 largest genera comprise nearly half of the species known from the country. Only two species are considered to be endemic: an unnamed *Anemia* and *Thelypteris mombachensis*. From the distributions maps, one can readily discern the most common (often weedy) ferns in Nicaragua: *Adiantum concinnum*, *Blechnum occidentale*, *Lygodium venustum*, *Microgramma percussa*, *Pityrogramma calomelanos*, *Tectaria heracleifolia* and *T. panamensis*, *Thelypteris dentata* (naturalized) and *T. nicaraguensis*. These, and a few others, are represented by more than 30 collections.

All species are estimated to fall into one of several categories depending on abundance/rarity of collections: in order of greatest endangerment these categories are CR, in critical danger; EN, in danger; VU, vulnerable; NT, somewhat threatened; LC, of lesser concern. Given the intrinsic uncertainties of assessing species vulnerability in any tropical area, approximately 35 spp. are considered as CR (usually only one collection known from the country); 160 spp. are EN (generally 1–2, up to ca. 7, collections known); and 156 spp. are VU (generally 4–10 collections known). By these estimates, more than 60% of the pteridophytes of Nicaragua are vulnerable, if not greatly threatened, a staggering percentage. Even though nearly all species of ferns have wider distributions outside the country, these statistics should cause concern. That

so many Nicaraguan ferns are known from only one or two collections also suggests that there are likely many species not yet collected in the country.

The illustrations are helpful, well executed, and pleasingly arranged by Alba Arbeláez, a co-author of the book. Kudos to her for her artistry, and for citing vouchers for the drawings! Also, the editing process is superb, the book is about as error-free as a flora can be. I enthusiastically recommend this book to anyone wanting to know about, or identify, pteridophytes from Nicaragua.—Alan R. Smith, University Herbarium, University of California, Berkeley, CA 94720-2465.

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QUARTERLY JOURNAL OF THE AMERICAN FERN SOCIETY

Comparative Photosynthetic Capacity of Abaxial and Adaxial Leaf Sides as Related to Exposure in Two Epiphytic Ferns in a Subtropical Rainforest in Northeastern Taiwan

Craig E. Martin, Rebecca (Chia-Chun) Hsu, and Teng-Chiu Lin **145**

Selected Physiological Responses of *Salvinia minima* to Various Temperatures and Light Intensities

Safaa H. Al-Hamdani and Jamil J. Ghazal **155**

Habitat Differentiation of Ferns in a Lowland Tropical Rain Forest

James E. Watkins, Jr. and Catherine Cardelús **162**

Eukaryotic Microbial Communities Associated with the Rhizosphere of the Temperate Fern *Thelypteris noveboracensis* (L.) Nieuwl.

O. Roger Anderson **176**

Structure and Organization of the Rhizome Vascular System of Four *Polypodium* Species

Archana Srivastava and Subhash Chandra **182**

***Isoetes maxima*, a New Species from Brazil**

R. James Hickey, C. Cecilia Macluf, and Melanie Link-Pérez **194**

New Records of *Polyphlebium borbonicum*, an African Filmy Fern, in the New World and Polynesia

Atsushi Ebihara, Joel H. Nitta, David Lorence, and Jean-Yves Dubuisson **200**

Aspects of Gametophyte Development of *Dicksonia sellowiana* Hook (Dicksoniaceae): an Endangered Tree Fern Indigenous to South and Central America

Cláudia Cristina L. Fiori, Marisa Santos, and Áurea M. Randi **207**

***In vitro* Study on Gametophyte Development of an Epiphytic Fern, *Arthromeris himalayensis* (Hook.) Ching, of South Sikkim, India**

Gautam Ganguly, Kaushik Sarkar, and Radhanath Mukhopadhyay **217**

An Efficient Method for Surface Sterilization and Sowing Fern Spores *in vitro*

Wu Hua, Chen Ping-Ting, Yuan Li-Ping and Chen Long-Qing **226**

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Comparative Photosynthetic Capacity of Abaxial and Adaxial Leaf Sides as Related to Exposure in Two Epiphytic Ferns in a Subtropical Rainforest in Northeastern Taiwan

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ABSTRACT.—Photosynthetic gas exchange was measured *in situ* with either the adaxial or abaxial leaf surface illuminated on vertical, horizontal, and angled leaves of *Asplenium nidus* and vertical leaves of *Ophioderma pendula*, two epiphytic ferns in a subtropical rain forest in northeastern Taiwan. Leaves for gas exchange measurements were selected to ensure a diversity of different exposures of the two leaf surfaces to direct sunlight. For most leaves of both species, photosynthetic rates were higher when the side of the leaf that typically received more direct insolation was illuminated during the gas exchange measurement. Higher rates of net CO_2 uptake when one side of the leaf was illuminated, relative to rates when the opposite side was illuminated, were attributable to a greater biochemical capacity for photosynthesis, not to greater stomatal conductances. Based on the results of this study, the photosynthetic capacity of the two sides of the leaves of epiphytic ferns, for the most part, reflects the degree of exposure of each side of the leaf to direct sunlight, as has been found in similar studies of terrestrial taxa.

KEY WORDS.—abaxial leaf surface, adaxial leaf surface, *Asplenium*, epiphytes, illumination, leaf angle, *Ophioderma*, photosynthesis, subtropical forest, Taiwan

Most leaves are green and, thus, presumably capable of some level of photosynthetic activity, even if just recycling respiratory CO_2, on both their adaxial and abaxial surfaces (Moore *et al.*, 1998; Terashima, 1986). Work with terrestrial taxa has shown that the capacity for photosynthesis is equal, or nearly so, when either leaf surface of vertically oriented leaves is illuminated, as long as both surfaces intercept similar amounts of solar radiation during leaf development (Syvertsen and Cunningham, 1979; DeLucia *et al.*, 1991; Poulson and DeLucia, 1993). In contrast, if one side of a vertically oriented leaf typically receives more insolation than the opposite side, the photosynthetic capacity of the leaf is greater when the normally sunlit surface is irradiated during photosynthetic measurements, relative to photosynthesis when the shaded side is irradiated (Poulson and DeLucia, 1993; but see Václavík, 1984)

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Likewise, the photosynthetic activity of horizontally oriented leaves is greater when their adaxial surface is illuminated than when their abaxial surface is illuminated (Syvertson and Cunningham, 1979; Terashima, 1986; DeLucia *et al.*, 1991) The latter applies only to the sun leaves, not the shade leaves, of Sitka spruce (Leverenz and Jarvis, 1979).

Epiphytic vascular plants appear to have been excluded from such studies, yet are ideal subjects for such investigations. Epiphytic vascular plants often exhibit a great diversity of leaf orientations and exposures (Benzing, 1990). For example, epiphytes with a rosette growth form often have leaves ranging from vertical to horizontal, and many have intermediate angles. Furthermore, a number of epiphytic taxa have vertically oriented leaves that are, unlike their terrestrial counterparts, positively geotropic. Most epiphytes also live in a complex light environment, being shaded by the host tree stem and canopy, as well as surrounding trees, depending on the location of the sun at any point in time. Given their leaf angles and the complexity of the light environment in which epiphytes grow, it is difficult to predict how the photosynthetic capacity of the two sides of the leaves of such plants compare and whether or not findings based on terrestrial taxa might apply to epiphytes. Therefore, the goal of this study was to determine if photosynthesis in epiphytes, particularly ferns, responds to leaf surface illumination in a similar manner as has been found in terrestrial plants.

Materials and Methods

Study site and species.—Leaf photosynthetic parameters were measured for six individuals of *Asplenium nidus* L. and five individuals of *Ophioderma pendula* (L.) Presl *in situ* at the Fushan Experimental Forest, a comparatively pristine tract of subtropical rainforest (121°34′E, 24°46′N) at an elevation of ~600 m located 40 km southeast of Taipei in northeastern Taiwan. For general climatic conditions at the Fushan site, see Martin *et al.* (2004). Environmental conditions during the week of measurements (11–15 July 2005) were: 25.1° C average daily air temperature (29.8° C average daily maximum; 21.3° C average daily minimum), 4.2 mbar average daily vapor pressure deficit (vpd); and 20.0 mol m^{-2} day^{-1} average daily Photosynthetic Photon Flux Density (PPFD).

Asplenium nidus and *O. pendula* were chosen for this investigation to ensure a diversity of different exposures of the two leaf surfaces to direct sunlight. Plants were selected in a partially disturbed section of the forest to allow easy access for *in situ* measurements of photosynthesis. The study site included several walking trails and was tens of meters from a laboratory building. Species of dominant trees at this site were numerous, primarily in the families Fagaceae and Lauraceae; examples include *Litsea acuminata* (Bl.) Kurata (Lauraceae), *Machilus zuihoensis* Hayata (Lauraceae), *Castanopsis cuspidata* (Thunb. *ex* Murray) Schottky var. *carlesii* (Hemsl.) Yamazaki (Fagaceae), and *Pasania hancei* (Benth.) Schottky (Fagaceae).

All plants were large (plant diameter for *A. nidus* ≥ 0.5m and leaf length for *O. pendula* ≥ 0.5m) growing epiphytically on a variety of host trees, including those listed above. Most plants had sporangia on some leaves at the time of this study (sporangia-bearing portions of the leaves were avoided in all measurements to avoid effects of sporangia on the measurements (Chiou *et al.* 2004). All leaves were measured no higher than three to four meters from the ground, i.e., within arm's reach while standing, with or without a ladder. Only mature, non-senescent leaves lacking substantial insect damage were sampled; very young and very old leaves were avoided. Leaves were selected without regard to host tree species, height from the ground (except as noted), and degree of canopy shade at the time of measurements.

Photosynthesis measurements.—Photosynthesis was measured on three different leaves for each of six plants of *A. nidus*; the three leaves were selected for measurements based primarily on the likelihood of exposure of each leaf surface to direct sunlight. Horizontal leaves were older (based on size, presence of sporangia, weathering, and phyllotaxy of the epiphyte) than the other two leaves selected for measurements and grew perpendicular to and away from the host tree trunk. Such leaves should intercept very little direct sunlight on their abaxial surface, whereas their adaxial surface should intercept direct sunlight during much of a sunny day. Angled leaves grew at about a 45 degree angle from the tree trunk, so should occasionally intercept direct sunlight on both surfaces of the leaf. Vertical leaves grew close to the trunk of the host tree, and, thus, were shaded by the trunk much of the day. These leaves should intercept little light on their adaxial surface most of the day, but occasionally direct sunlight on their abaxial surface, depending on the location of the sun. All leaves of *Ophioderma pendula* grew with similar exposure to light on their two surfaces as in the "vertical" leaves of *A. nidus*, but, in contrast to leaves of *A. nidus*, the growth of *O. pendula* leaves was positively geotropic. Another important difference between the "vertical" leaves of these two epiphytic ferns is that the leaf sides are reversed in the two taxa, i.e., in *A. nidus*, the abaxial side of the "vertical " leaves faces outward, and is thus more exposed to solar irradiation, whereas, the adaxial surfaces of the *O. pendula* leaves face outward and are thus more exposed to direct sunlight. Although intercepted sunlight on all leaves and their two surfaces was not measured, field observations during this study confirmed the above statements.

Photosynthesis was measured with a LI-COR (Lincoln, NE) LI-6400 Portable Photosynthesis System. Because all leaves measured were large, the area of leaf for which gas exchange was measured matched the maximum area possible (6 cm^2) in the gas exchange chamber. Photosynthetic parameters were measured two different ways at the central portion of each leaf: once with the adaxial surface illuminated and again adjacent to the same leaf location with the abaxial surface illuminated. The exact same location on the leaf was not used for both measurements to ensure that manipulation by inserting the leaf into the chamber and clamping the chamber on the leaf for the first measurement did not influence the second measurement. Although gas

exchange was always measured for both sides of the leaf simultaneously, the chamber was oriented such that only the adaxial or abaxial surface received light from the blue and red diodes in the top half of the chamber. Very little ambient light reached the opposing leaf surface during the measurements as a result of shading by parts of the gas exchange chamber, the investigators, and nearby vegetation. For both species, photosynthesis was measured three times with illumination on one surface of a leaf at a low PPFD (100 μmol m^{-2} s^{-1}), then three times at a high PPFD (1000 μmol m^{-2} s^{-1}). Using the same leaf, the chamber was then reversed to measure gas exchange with illumination (both PPFD levels) on the opposite leaf surface. Net CO_2 uptake in *A. nidus* saturated at approximately 500 μmol m^{-2} s^{-1} (determined with preliminary gas exchange measurements). Other environmental conditions during all measurements were maintained by the LI-6400 system at the following values: air CO_2 concentration of 370 μmol mol^{-1}, chamber ("block") temperature of 30°C (leaf temperatures were typically 0.5° C higher), vapor pressure deficit (vpd) of 0.9 mbar, and flow rate of 200 μmol s^{-1}. Lower vpd values resulted in exceedingly low transpiration rates, which led to unrealistic values for C_i; any such data were discarded. For each gas exchange measurement, data were recorded only when gas exchange rates were stable (Coefficient of Variation of exchange rates of both gases and flow rates not varying by more than 0.2% among successive measurements every 2–3 seconds), typically within 10 seconds of inserting the leaf in the gas exchange chamber or after the previous measurement (for a total of three repeated measurements). The gas exchange chamber remained clamped to a leaf for approximately five minutes at each light level, allowing for stable readings, as well as steps taken to ensure instrument accuracy (e.g., using the "match" function of the LI-6400 prior to each measurement).

Statistical analyses.—For both species, means (N=5 or 6 plants; the value for each plant being a mean of three repeat measurements; see above) of abaxial and adaxial gas exchange parameters at each light level were compared with a paired Student's *t*-test when the data met the assumptions of parametric statistics (Sokal and Rohlf, 1981) or with a Mann-Whitney *U*-test otherwise.

Results and Discussion

The adaxial side of the vertical leaves growing out of the rosettes of *A. nidus* is unlikely to receive direct radiation due to shading by the host tree trunk, whereas the exposed abaxial side should at least occasionally intercept direct solar radiation. Thus, based on results with terrestrial plants (Syvertson and Cunningham, 1979; Terashima, 1989; DeLucia *et al.* 1991; Poulson and DeLucia 1993), it was predicted that the illumination of the abaxial side of the vertical leaves of *A. nidus* would result in higher photosynthetic rates than when the adaxial side of the same leaf is illuminated. Measurements of photosynthesis at both high and low PPFD of plants in northeastern Taiwan did not, however, support this prediction (Fig. 1). In contrast, although not statistically significant (high PPFD P= 0.28; low PPFD P = 0.17), the trend in

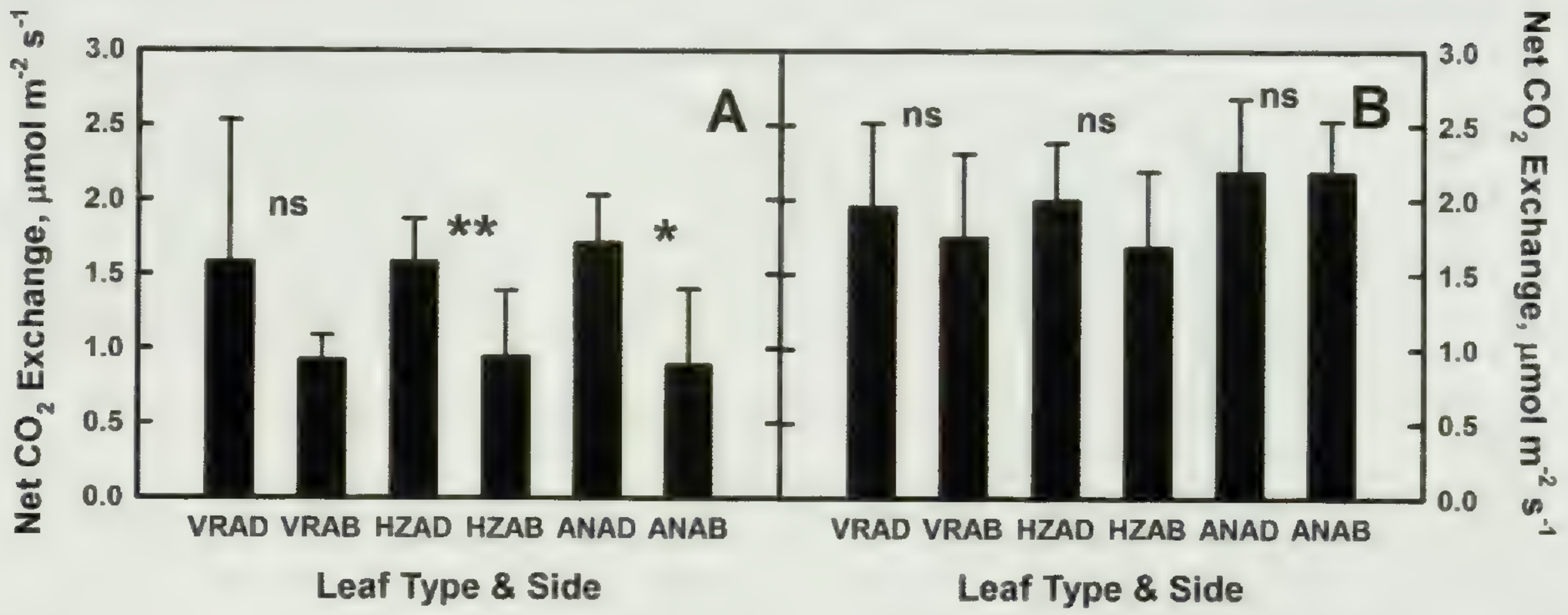

FIG. 1. Mean (lines projecting from bars are standard deviations; n = 6 plants, three repeated measurements/leaf/plant) rates of net CO_2 exchange (positive values indicate CO_2 uptake) for different leaves and with illumination at two light levels on either side of the leaves of the epiphytic fern *Asplenium nidus* measured *in situ* in a subtropical rain forest in northeastern Taiwan). Abbreviations for type and side of leaf are: "VR" = vertical, "HZ" = horizontal, "AN" = angled (45° from vertical); and "AD" indicates illumination (A, 100 μmol m^{-2} s^{-1}; B, 1000 μmol m^{-2} s^{-1}) provided to the adaxial side of the leaf during gas exchange measurements; "AB" indicates illumination (low and high PPFD as in AD) provided to the abaxial side of the leaf during measurements. The abaxial and adaxial means for two leaves at low PPFD are significantly different at $P < 0.05$ or $P < 0.01$ indicated by "*" or "**", respectively, above each pair of means, while the other pairs of means are not significantly different ($P > 0.05$, indicated by "ns" above each pair of means).

the data indicated the opposite of expectations, i.e., photosynthetic rates at either PPFD appeared higher when the adaxial surface was illuminated. According to the statistical analyses, however, photosynthetic rates at both light levels were equal regardless of which side of the leaf was illuminated (Fig. 1).

Light interception of the two surfaces of the horizontal leaves of the epiphytic fern *A. nidus* is quite different from that of the vertical leaves, and the prediction of comparative photosynthetic capacities when the two sides of this leaf are illuminated is the opposite of that of the vertical leaves of this fern. Because the adaxial surfaces of these leaves intercept more direct solar radiation than do the abaxial surfaces, photosynthetic rates when the adaxial surface of the horizontal leaves of this epiphyte are illuminated should be higher than those of the leaf when the abaxial surface of the same leaf is illuminated. Measurements of photosynthetic rates confirmed this prediction, although the higher photosynthetic rates when the adaxial side of the leaves was illuminated were statistically significant only when measurements were made at the lower PPFD (Fig. 1). These higher net CO_2 uptake rates were accompanied by equal transpiration rates (Fig. 2) and stomatal conductances (Fig. 3), while internal CO_2 concentrations were significantly lower (Fig. 4) These gas exchange results indicate that the higher photosynthetic rate was most likely the result of a greater biochemical capacity for photosynthesis and not the result of greater stomatal opening and, hence, easier gas diffusion into

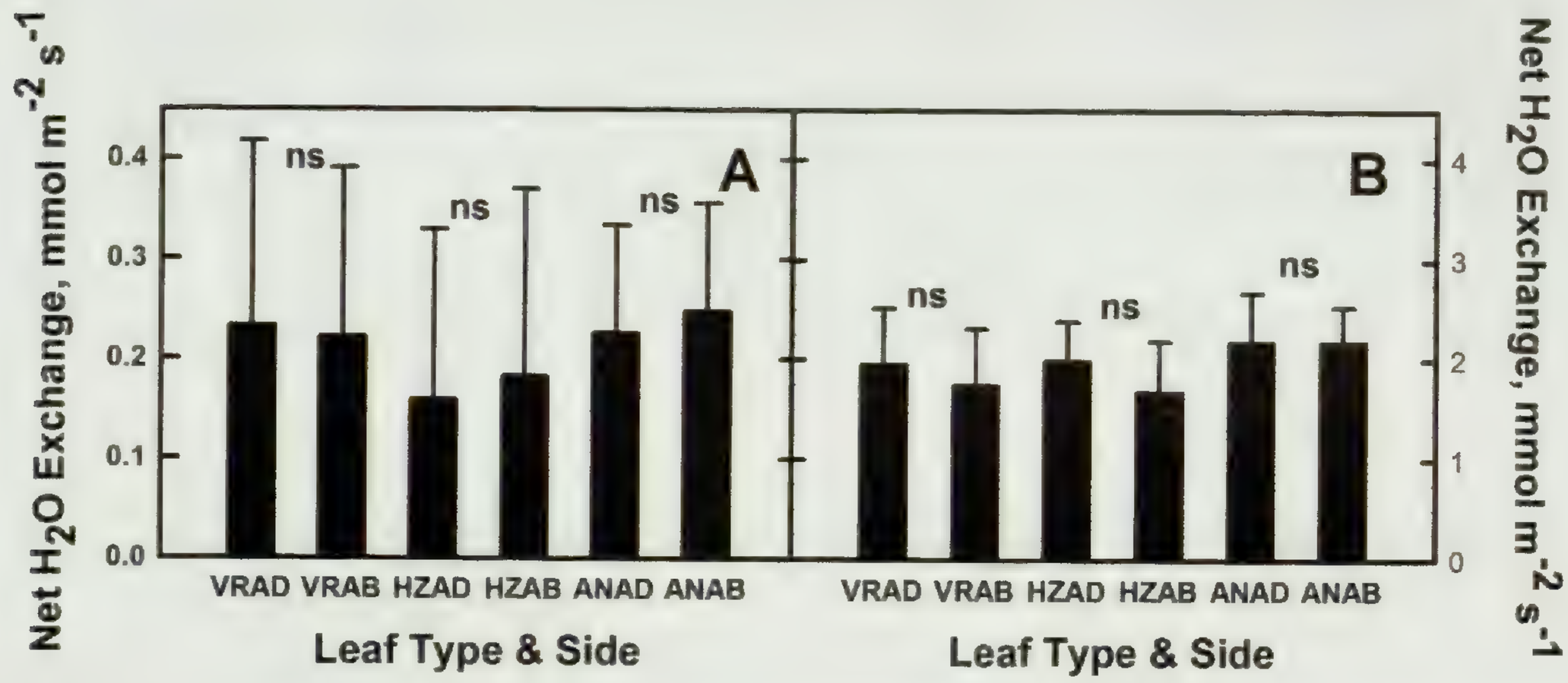

FIG. 2. Mean (lines projecting from bars are standard deviations; n = 6 plants, three repeated measurements/leaf/plant) rates of net H_2O exchange (positive values indicate water vapor loss) for different leaves and with illumination at two light levels on either side of the leaves of the epiphytic fern *Asplenium nidus* measured *in situ* in a subtropical rain forest in northeastern Taiwan). Abbreviations for type and side of leaf are: "VR" = vertical, "HZ" = horizontal, "AN" = angled (45° from vertical); and "AD" indicates illumination (A, 100 μmol m^{-2} s^{-1}; B, 1000 μmol m^{-2} s^{-1}) provided to the adaxial side of the leaf during gas exchange measurements; "AB" indicates illumination (low and high PPFD as in AD) provided to the abaxial side of the leaf during measurements. None of the abaxial and adaxial means at any leaf location are significantly different ($P > 0.05$, indicated by "ns" above each pair of means).

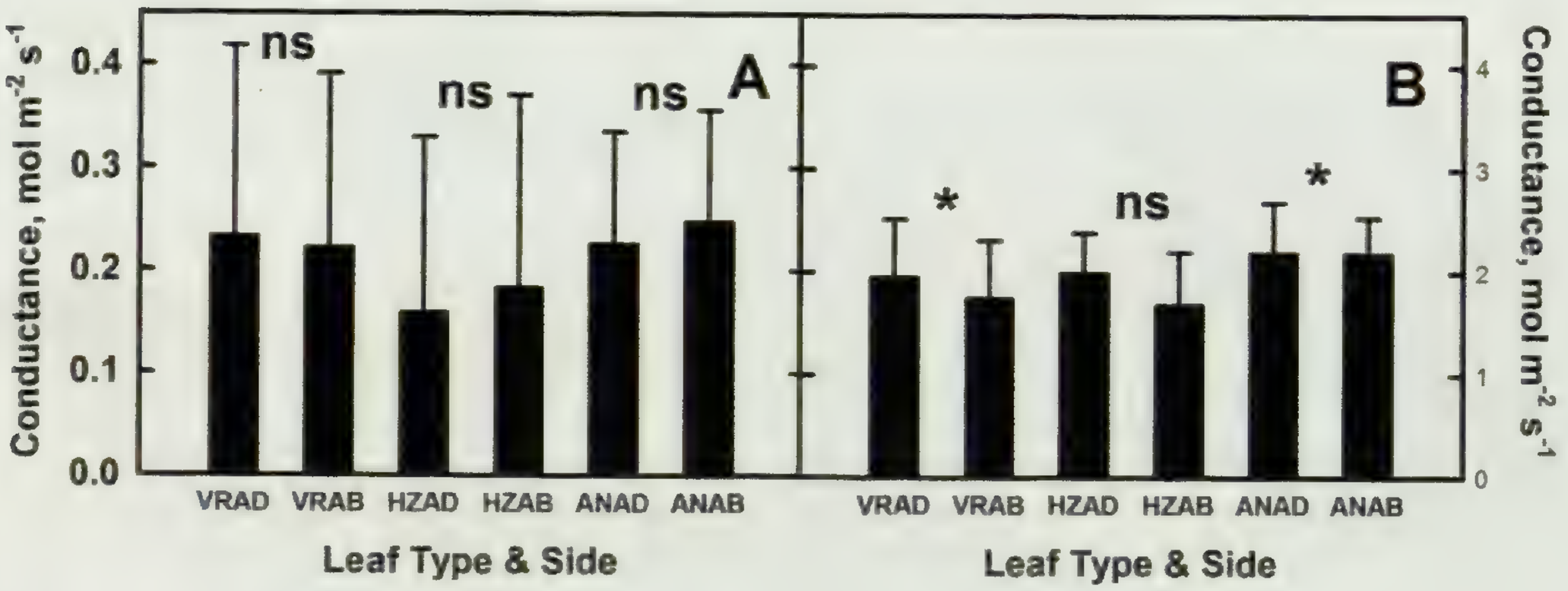

FIG. 3. Mean (lines projecting from bars are standard deviations; N = 6 plants, three repeated measurements/leaf/plant) stomatal conductances for different leaves and with illumination at two light levels on either side of the leaves of the epiphytic fern *Asplenium nidus* measured *in situ* in a subtropical rain forest in northeastern Taiwan). Abbreviations for type and side of leaf are: "VR" = vertical, "HZ" = horizontal, "AN" = angled (45° from vertical); and "AD" indicates illumination (A, 100 μmol m^{-2} s^{-1}; B, 1000 μmol m^{-2} s^{-1}) provided to the adaxial side of the leaf during gas exchange measurements; "AB" indicates illumination (low and high PPFD as in AD) provided to the abaxial side of the leaf during measurements. The abaxial and adaxial means at two leaf locations at high PPFD are significantly different at $P < 0.05$, indicated by "*" above each pair of means, while the other pairs of means are not significantly different ($P > 0.05$, indicated by "ns" above each pair of means).

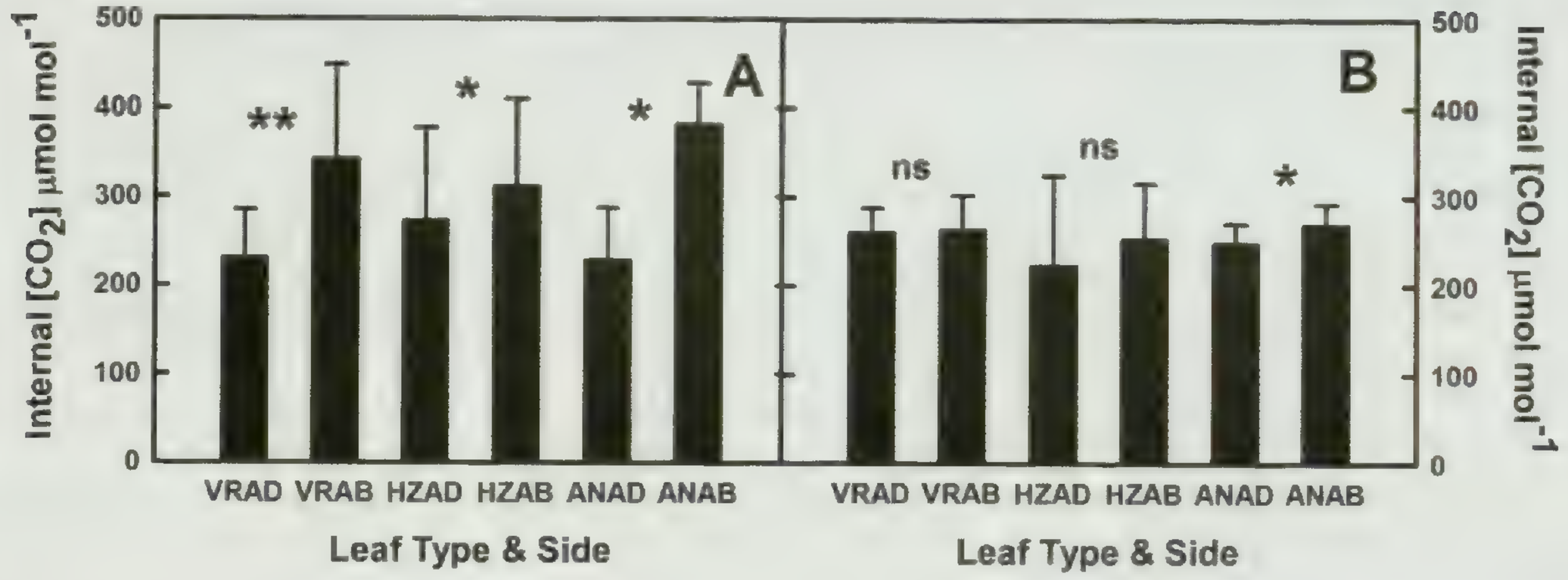

FIG. 4. Mean (lines projecting from bars are standard deviations; N = 6 plants, three repeated measurements/leaf/plant) leaf internal CO_2 concentrations (external CO_2 concentration was 370 µmol mol^{-1}) for different leaves and with illumination at two light levels on either side of the leaves of the epiphytic fern *Asplenium nidus* measured *in situ* in a subtropical rain forest in northeastern Taiwan). Abbreviations for type and side of leaf are: "VR" = vertical, "HZ" = horizontal, "AN" = angled (45° from vertical); and "AD" indicates illumination (A, 100 µmol m^{-2} s^{-1}; B, 1000 µmol m^{-2} s^{-1}) provided to the adaxial side of the leaf during gas exchange measurements; "AB" indicates illumination (low and high PPFD as in AD) provided to the abaxial side of the leaf during measurements. The abaxial and adaxial means at several leaf locations are significantly different at $P < 0.05$ or $P < 0.01$, indicated by "*" or "**", respectively, above each pair of means, while the other pairs of means are not significantly different ($P > 0.05$, indicated by "ns" above each pair of means).

the leaf (Farquhar and Sharkey, 1982; Sharkey, 1985). In agreement with the latter interpretation, it is possible, especially for the measurements made at high light, that illumination of the abaxial surface resulted in photoinhibition in this lateral half of the section of leaf being measured. This possibility is supported by previous findings that the side of a leaf that is typically less exposed to sunlight has chloroplasts and photosynthetic features typical of shade-adapted leaves (Schreiber *et al.*, 1977; Kulandaivelu *et al.*, 1983; Terashima and Inoue, 1984; Terashima *et al.*, 1986). Differences in photosynthetic capacity depending on which side of the leaf is illuminated might also reflect other anatomical or optical (e.g., absorptance) features of the two sides of the leaf (Terashima 1986; DeLucia *et al.*, 1991). Such differences would also be interpreted as non-stomatal and non-diffusional mechanisms responsible for differences in photosynthesis between the two sides of the leaf, as was found in this study.

Both the adaxial and abaxial surfaces of the "angled" leaves of *A. nidus* should intercept direct sunlight, at least for brief periods, throughout a day. Thus, one might predict that the photosynthetic capacity of these leaves is comparable, regardless which surface is illuminated (Syvertsen and Cunningham, 1979; Václavík, 1984; DeLucia *et al.*, 1991; Poulson and DeLucia, 1993). Based on measurements made *in situ* with this epiphytic fern in northeastern Taiwan, this prediction was supported when gas exchange was measured at high PPFD (Fig. 1), but the photosynthetic rate when the adaxial leaf surface

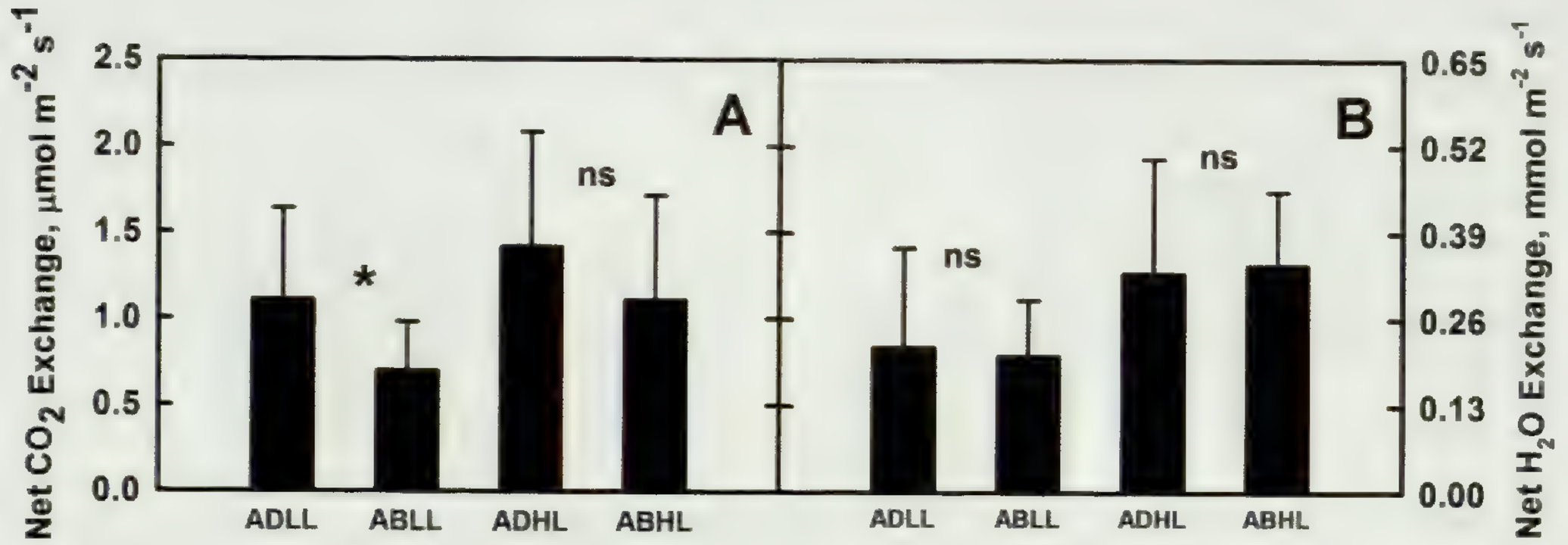

FIG. 5. Mean (lines projecting from bars are standard deviations; n = 5 plants, three repeated measurements/leaf/plant) rates of net CO_2 exchange (A; positive values indicate CO_2 uptake) and rates of net water vapor exchange (B; positive values indicate water vapor loss) with illumination at two light levels on either side of the leaves of the epiphytic fern *Ophioglossum pendula* measured *in situ* in a subtropical rain forest in northeastern Taiwan). Abbreviations for side of leaf and light level are: "AD" indicates illumination (LL = 100 µmol m^{-2} s^{-1}; HL= 1000 µmol m^{-2} s^{-1}) provided to the adaxial side of the leaf during gas exchange measurements; "AB" indicates illumination (low and high PPFD as in AD) provided to the abaxial side of the leaf during measurements. The abaxial and adaxial means at high PPFD are significantly different at $P < 0.01$, indicated by "*" above that pair of means, while the other pairs of means are not significantly different ($P > 0.05$, indicated by "ns" above that pair of means).

was illuminated exceeded that when the abaxial surface of the leaf was illuminated at low PPFD (Fig. 1). As was the case with the horizontal leaves, the higher net CO_2 exchange rate of the angled leaves was apparently the result of a greater biochemical capacity for photosynthesis, generating a lower leaf internal CO_2 concentration (Fig. 4), and not due to a greater stomatal conductance (Fig. 3; Farquhar and Sharkey, 1982; Sharkey, 1985). These findings contrast directly with those for Sitka spruce by Leverenz and Jarvis (1979), who found that differences in photosynthetic capacity of the leaves, depending on which side of the leaf was illuminated could be ascribed to differences in stomatal conductance, not to the biochemical capacity of the leaf.

The leaves of *Ophioderma pendula* are positively geotropic, hanging vertically from the main body of this epiphytic fern and remaining close to the main stem of the host tree. As a result of shading by the immediately adjacent host tree trunk, the abaxial surfaces of these leaves seldom receive direct solar radiation. Thus, it was predicted that photosynthetic rates, at least at the higher PPFD, when the adaxial surface of these leaves is illuminated, would exceed those when the abaxial surfaces of these leaves are illuminated for plants measured *in situ* in this subtropical rain forest. This was indeed the case for net CO_2 exchange measurements at both high and low PPFD (Fig. 5). Because rates of transpiration and stomatal conductances were equal when either side of the leaves was illuminated during gas exchange measurements

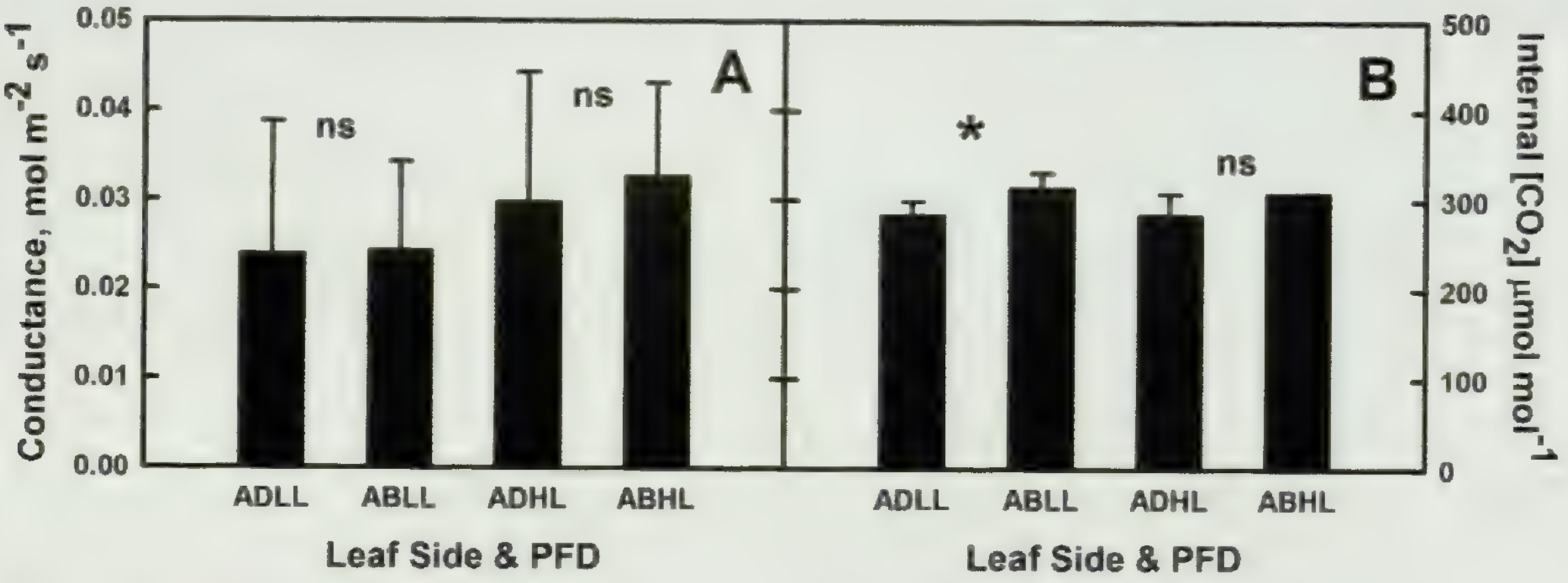

FIG. 6. Mean (lines projecting from bars are standard deviations; n = 5 plants, three repeated measurements/leaf/plant) stomatal conductances (A) and leaf internal CO_2 concentrations (B; external CO_2 concentration was 370 µmol mol^{-1}) with illumination at two light levels on either side of the leaves of the epiphytic fern *Ophioglossum pendula* measured *in situ* in a subtropical rain forest in northeastern Taiwan). Abbreviations for side of leaf and light level are: "AD" indicates illumination (LL = 100 µmol m^{-2} s^{-1}; HL= 1000 µmol m^{-2} s^{-1}) provided to the adaxial side of the leaf during gas exchange measurements; "AB" indicates illumination (low and high PPFD as in AD) provided to the abaxial side of the leaf during measurements. Neither pair of abaxial and adaxial means is significantly different ($P > 0.05$, indicated by "ns" above the pairs of means).

(Figs. 5, 6), and because internal CO_2 concentrations were lower (although not statistically significantly so at high PPFD; Fig. 6) when the adaxial surface was illuminated, the mechanism underlying the higher rate of net CO_2 uptake when the adaxial surface was illuminated appears, as was the case in several instances with *A. nidus*, to reflect a greater biochemical capacity for photosynthesis, not a greater stomatal conductance allowing easier CO_2 diffusion into the leaf (Farquhar and Sharkey, 1982; Sharkey, 1985).

Overall, the results of *in situ* gas exchange measurements with two epiphytic ferns in a subtropical rain forest in northeastern Taiwan lend considerable, but not complete, support to past findings with terrestrial taxa (Syvertsen and Cunningham, 1979; Terashima, 1989; DeLucia *et al.*, 1991; Poulson and DeLucia, 1993). In most, but not all, cases, if a leaf is oriented such that one side receives more direct solar radiation than the other, the leaf has a higher photosynthetic capacity when the more exposed surface is illuminated. In addition, this higher capacity reflects a greater biochemical capacity for photosynthesis and not easier diffusion of CO_2 into the leaf (Farquhar and Sharkey 1982; Sharkey, 1985).

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Selected Physiological Responses of *Salvinia minima* to Various Temperatures and Light Intensities

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ABSTRACT.—Two separate experiments were conducted to determine the influence of temperature (15, 23 and 35 °C) and various light intensities, ranging from 80 to near 700 μmol/m^2/s on selected physiological responses of salvinia (*Salvinia minima*). This was an attempt to determine the distribution range of this plant as influenced by these selected environmental factors. The first experiment was carried out for 14 days under controlled environments, with a light intensity of 120 μmol/m^2/s and 14 h photoperiod. Plant growth was the highest at 23 and 35 C, in comparison to those grown at 15 °C. The chlorophyll concentration was less influenced by the temperature than by the growth; however, carotenoid concentration at 35 C was significantly higher than those obtained from the plant grown at 15 C. Salvinia acclimation to cold temperature possibly included an increase in athocyanin and soluble sugar concentrations. The second experiment was carried out under greenhouse conditions, 25–27 C and various light intensities ranging between 80 to near 700 μmol/m^2/s in order to determine the light saturation curve. Salvinia was shown to have a wide range of acclimation ability to various light intensities ranging from 80 to near 700 μmol/m^2/s. This study should be helpful for determining the ecological distribution of salvinia.

KEY WORDS.—*Salvinia minima*, CO_2 assimilation, photosynthetic pigments, light intensities

The genus *Salvinia*, from the family Salviniaceae, is comprised of 12 known species (Nauman, 1993). *Salvinia minima* Baker is a small, free-floating freshwater fern found in tropical and temperate regions (DeBusk and Reddy, 1987) and in areas such as North, South, and Central America, the West Indies, and Africa (Nauman, 1993). This species is commonly referred to as water fern and South American pond fern (Nauman, 1993). In the United States, salvinia was first discovered in the St. John's River, Florida in 1928 (Long and Lakely, 1976) and was speculated to have been introduced by accident through its discharge from contaminated boat ballasts from South American ships or from accidental release from aquarium sources (Schmitz *et al.*, 1991). This plant can be found floating near the edges of slow moving streams and in nutrient enriched ponds. Salvinia forms a "mat" that covers very large portions of the body of water where it grows. Salvinia reproduces exponentially by vegetative fragments, with a leaf doubling time of 3.5 days (Nichols *et al.*, 2000). The rapid growth of salvinia has been recognized as an ecological problem in many southern coastal regions of the United States. This is due to the impact on suppressing the growth of native vegetation and the degradation of water quality; this includes reducing oxygen concentration, reduces the light

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penetration, and other ecological impacts (McFarland *et al.*, 2004). However, The unique characteristics of salvinia, which include rapid growth, the high acclimation capacity of the plant to wide range of temperatures, and relative tolerance to a wide range of contaminants make it a prime candidate for phytoremediation (Olguin *et al.*, 2003; Olguin *et al.*, 2007). For instance, *Salvinia minima* demonstrated the ability to withstand aluminum (Al) concentrations of 20 mg/l through the manipulation of the media pH from 3.9 to near 7 within 24 hours (Gardner and Al-Hamdani, 1997). In addition, salvinia has the ability to survive and grow under highly eutrophic environments unsuitable for other species found in similar environments such as azolla (*Azolla caroliniana* Willd.) and duckweed (*Lemna minor* L.) (Reddy and DeBusk, 1985).

Whether salvinia is perceived as either a noxious or beneficial weed, it is essential to determine the environmental requirements for its growth. Temperature and light intensity are among major environmental factors determining the ecological distribution of any plant (Larcher, 2003). There is a lack of general information regarding these environmental requirements for salvinia growth. Therefore, in this study, the influence of selected temperatures (15, 25, 35°C) on salvinia growth was determined. To examine the ability of salvinia to combat the impact of low temperature, the accumulation of soluble sugar was examined. The accumulation of soluble sugar is one of the major defense mechanisms of plants to cold temperatures (Larcher, 2003). In addition, the photosynthetic light response curve was determined to evaluate the photosynthetic response of salvinia to different light intensities. The photosynthetic pigment concentrations at different temperatures were evaluated and anthocyanin concentration was also determined. Anthocyanin is a major flavonoid pigment which is considered a free radical scavenger reducing the damages resulting from oxidative stress such as photooxidation (de Pascual-Teresa and Sanchez-Ballesta, 2007).

Materials and Methods

To accomplish the objective of this study, several separate experiments were carried out under controlled environments of temperature and light intensity. To determine the effect of temperature (15, 23, 35°C) on salvinia growth, a total of 20 fronds were placed into 250 ml Erlenmeyer flasks containing 125 ml of 10% Hoagland's solution (Hoagland and Arnon, 1938). The plants utilized in this study were taken from stock material growing under greenhouse conditions. The plants of all treatments were grown for fourteen days in a growth chamber at 23°C, 120 $\mu mol/m^2/s$ photon flux density and 14 h photoperiod. Twelve flasks (samples) per treatment were used. The samples of each treatment were placed in separate containers. Water was added to each container, and the flasks were partially submerged in a water bath. The individual temperatures were controlled by passing water with the desirable temperature from a circular water bath unit through cooper coils located inside the individual water containers.

The plants of six samples (flasks) from each treatment were used for growth determination. The remaining six samples of each treatment were used to determine the soluble sugar content. Salvinia growth was determined using fresh weight and frond number doubling time. Frond number of each sample was counted at the initial day of the experiment (day1) and on day 7 and 14. The frond numbers were used to evaluate the growth rate using the following doubling time formula: $DT = t \log 2 [\log (w_t w_o^{-1})]^{-1}$ (Moretti and Gigliano, 1988) where DT is the doubling time (days), t is the experiment duration (days), w_t is the final number of frond, and w_o is the initial frond number. Approximately 0.1 g fresh weight of each sample was used for measuring chlorophyll *a* and *b*, and carotenoid concentration. The plants were placed into 5 ml of N,N-Dimethylformamide (DMF) solution. The samples were incubated in the dark for 36 hrs at 4°C. Chlorophyll *a* and *b* was determined spectrophotometrically at wavelengths of 647 and 664.5 nm (Inskeep and Bloom, 1985). The anthocyanin was determined by homogenizing 0.1 g fresh tissues in 5ml methanol containing 1% HCl (v/v) for 2 min on ice. The homogenate was filtered and absorbance of the extract was determined spectrophotometrically by the method of Mancinelli (1990).

Soluble sugar analysis was conducted following a procedure slightly modified from Chatterton *et al.* (1987). Six randomly selected samples of each treatment were oven dried at 65°C for 48 h. The dry samples were ground into a fine powder, and a 100–500 mg portion was placed in a sealed vial and used to measure soluble sugars as reported in detail by Wilson and Al-Hamdani (1997).

This experiment was repeated twice each and statistically analyzed as a randomized complete block design (Steel and Torrie, 1980). This design ensured that observed differences in plant performances were largely due to treatments rather than variation among the four blocks (experiments conducted at different times). Mean separations for the treatments with significant F values ($P = 0.05$) of ANOVA analysis were based on the least significant difference (LSD) test (Steel and Torrie, 1980).

The second experiment was carried out to determine the photosynthetic light response curve. The salvinia was grown in Hoagland's solution, as in the first experiment. Six flasks containing 20 fronds of salvinia were grown for 7 days under greenhouse conditions of 25–27°C and various light intensities, depending on the time of day, ranging from 80 to near 700 $\mu mol/m^2/s$.

Carbon dioxide assimilation of the six samples was measured starting four hours after the onset of the light period at day seven. The selected plants of each sample were placed on wet filter paper and enclosed in a flow-through plexiglass assimilation chamber (4.5 by 11.8 by 7.3 cm) of a Li-Cor 6200 photosynthesis system (Lincoln, NE, USA) as described by McDermitt *et al.* (1989). Standard measurement conditions were 27°C, various light intensity with ranges from 80 to near 700 μmol $\mu mol/m^2/s$ photon flux density and 75% relative humidity. To establish a light saturation curve, photosynthesis measurements were taken at various times of the day to obtain the desired range of light intensities. This experiment was repeated three times.

TABLE 1. The effect of different temperatures on salvinia growth and soluble sugar accumulation.

Temperature (°C)	Doubling time (days)		Soluble sugar* mg/g dry weight
	Length of exposure (days)		
	7	14	
15	−33.559a	53.364a	78.130a
23	3.865b	4.668bb	33.830b
35	3.468b	7.741b	14.953b

Mean within the column followed by the same lower case letter are not significant based on LSD (P = 0.05).

* The soluble sugar was determined after fourteen days of exposure at the selected temperatures.

RESULTS AND DISCUSSION

Assessment of salvinia growth at the three selected temperatures clearly showed that exposure to colder temperatures (15°C) resulted in significant growth decline in contrast to samples exposed to higher temperatures (Table 1). After seven days of growth at 15°C, salvinia doubling time showed a negative value, indicating no determinable change in the growth status during that period. This result was expected since salvinia is a tropical plant and is susceptible to cold temperatures (Gaudet, 1973). Debusk and Reddy, 1987 reported that the growth rate of *Salvinia rotundifolia* was significantly lower at 10°C, compared to samples grown at 25°C. The optimum growth temperature for most tropical plant species was reported between 23 to 32°C. (Lee *et al.*, 2007). Salvinia grown at 23 and 35°C showed similar rapid growth, as indicated by the doubling time values, which was less than four days (Table 1). The growth results after 14 days were comparable to those at seven days. The exception were the plants grown at 15°C, which demonstrated very slow growth rate, with a positive value for the doubling time (53.36 days). This result indicates that salvinia can survive at low temperature of 15°C.

Chlorophyll *a* and *b* concentrations were similarly influenced by the three temperature treatments (Table 2). The exception was plants grown at 35°C which showed significantly higher chlorophyll *a* concentration than the other temperatures. Similar findings were reported by McWilliam and Naylor (1967). They reported a reduction in chlorophyll content in corn (*Zea mays* L.) associated with lowering the growth temperature from 28 to 16°C. The commonly observed reduction in chlorophyll concentration in tropical plants at low temperature was attributed in part to an aberrant development of the thylakoid membranes (Hodgins and van Huystee, 1986). In addition, low temperature was found to induce a reduction in several enzymes associated with chlorophyll synthesis in the plant (Tewari and Tripathy, 1998). In this study, the temperature treatments of 35°C induced significant increases in carotenoid concentration in comparison to those grown at 15°C (Table 2). However, temperature effect of 23°C on carotenoid concentration was not significantly different to those grown at 15 and 35°C. Lefsrud and Kopsell (2005) reported an increase in β-Carotene concentration in kale (*Brassica*

TABLE 2. The influence of selected temperatures on chlorophyll a (chl a), chlorophyll b (chl b), carotenoid and anthocyanin concentrations in salvinia after fourteen days of growth.

Temp. (°C)	Chl a (mgg^{-1} fr.wgt.)	Chl b (mgg^{-1} fr.wgt.)	Carotenoid (μgg^{-1} fr.wgt)	Anthocyanin (μgg^{-1} fr.wgt)
15	6.163a	3.747a	515.075a	31.480a
23	6.762a	4.235a	686.625ab	10.478b
35	8.903b	4.963a	1252.452b	21.698c

Mean within the column followed by the same lower case letter are not significant based on LSD (P = 0.05).

oleracea L.) in response to gradual temperature increases from 15, 20, 25 to 30°C. Similarly, Leipner *et al.* (1997) found a decline in chlorophyll *a* and chlorophyll *b* and carotenoid concentrations in corn associated with lowering the temperature from 25 to 15°C.

Anthocyanin concentration was significantly different among the plants grown at different temperatures (Table 2). The highest anthocyanin concentration was obtained from the plant grown at 15°C, followed in decreasing order by those grown at 35 and 23°C. The increase in anthocyanin concentration in response to the relatively low (15°C) and high (35°C) temperatures might be considered an acclimation response to stress. This conclusion was also supported by Doong *et al.* (1993), who reported that anthocyanins are produced by most aquatic plants in response to stress factors such as high light intensity, high temperature, or nutritional limitations, and can be used as a stress indicator. Increased anthocyanin concentrations were also found to be induced in azolla by Al stress (Ayala-Silva and Al-Hamdani, 1997) and by Cr (VI) (Wilson and Al-Hamdani, 1997).

Salvinia accumulation of soluble sugar was significantly increased with each decrease in temperature from 35 to 23 and 15°C (Table 1). This increase in soluble sugar might represent an acclimation response to low temperature. Sugar accumulation could play an important role in combating the influence of low temperature (Levitt, 1980; Hurry *et al.*, 1995). Similar findings were reported by Al-Hamdani and Thomas (2000). Strand *et al.* (1997) suggested that an increase in soluble sugar accumulation is an essential response to combat the cold temperature stresses. The advantage of increasing soluble sugar is associated with the reduction in freezing point and increase plant tolerance to cold temperature (Nilsen and Orcutt, 1996).

The connection between light intensity and photosynthesis showed that the light-limiting portion of the light saturation curve extended to near 300 $\mu mol/m^2/s$ (Fig. 1). This can be used as an indicator that photosynthesis was operating linearly with the light intensity during this portion of the curve. The CO_2 limiting portion of the curve extended from 350 to near 700 $\mu mol/m^2/s$. Similar findings were reported for Floating Pennywort (*Hydrocotyle ranunculoides* L.f.) with a light saturation of CO_2 gas exchange between near 350 to near 800 $\mu mol/m^2/s$ (Hussner and Losch, 2007).

In conclusion, salvinia growth was the highest at 23 and 35°C and lowest at 15°C. However, the reduction in plant growth was not severe enough to totally

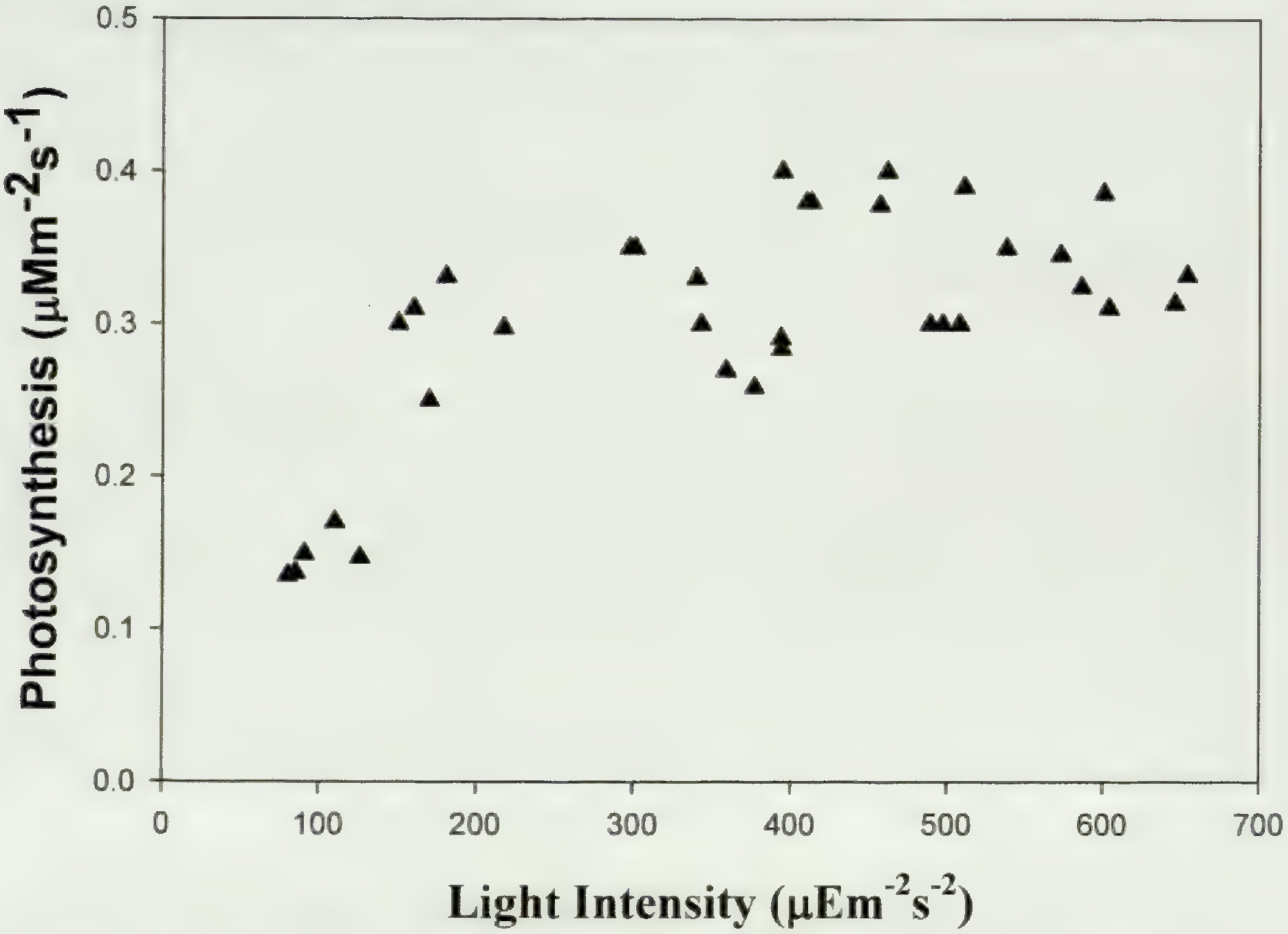

FIG. 1. The relationship between photosynthesis and light intensity for *Salvinia minima*.

inhibit plant reproduction. Salvinia acclimation to cold temperature possibly included the increase in anthocyanin and soluble sugar concentrations. The light saturation curve indicated that salvinia has a wide range of acclimation to various light intensities ranging from 80 to near 700 μmol/m^2/s and should be helpful for determining the ecological distribution of salvinia. Since one of the major criteria in selecting plants for ecological restoration is the acclimation capacity to the range of temperature and light intensity (Salt *et al.*, 1998), salvinia's capability to withstand a diverse range of environmental variables make it a prime candidate for phytoremediation.

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Habitat Differentiation of Ferns in a Lowland Tropical Rain Forest

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ABSTRACT.—Fern species and growth form diversity peak in tropical rainforests. In such forests, ferns often play important ecological roles. However the distribution and diversity patterns of different growth forms (i.e., epiphytic vs. terrestrial ferns) have not been broadly quantified. We compared the distribution and diversity patterns of epiphytic pteridophytes on the trunks of six individuals of the emergent canopy tree species *Hyeronima alchorneoides* (Euphorbiaceae) to those of terrestrial species at La Selva Biological Station in Heredia province, Costa Rica. A total of 21 species of epiphytic and 20 terrestrial ferns was recorded, with only one species found as an epiphyte and as a terrestrial species. Epiphytic species also exhibited increasing species diversity with increasing trunk height. Epiphytic species exhibited predictable patterns of distribution along the trunk and were easily grouped into high-trunk, low trunk, or bimodal categories. In terms of percent cover and number of species, simple-leaved ferns dominated the epiphytic growth form, 13 of 21 species, whereas ferns with compound or dissected leaves dominated the hemi-epiphytic and terrestrial floras with 20 of 20 species. These results indicate that there are significant functional differences in the ecology of epiphytic and terrestrial ferns and that reciprocal establishment is difficult and extremely rare.

KEY WORDS.—Pteridophyte, epiphyte, canopy, microclimate, gametophyte

Pteridophytes, especially the ferns, make up an important component of tropical and temperate floras and serve important functions in ecosystem processes in both the canopy (Hietz, 1997) and forest floor habitats (Hill and Silander, 2001). Epiphytic ferns make up an especially conspicuous component of tropical wet forest regions around the world. For example, in Costa Rica, 70% of the entire pteridoflora is epiphytic, while at La Selva Biological Station in northeastern Costa Rica epiphytic ferns comprise 42% of this lowland forest flora (Grayum and Churchill, 1987). Surprisingly, our understanding of the ecology of epiphytic taxa is especially limited.

Recent studies on the comparative biology of epiphytic and terrestrial fern species have revealed significant differences in the gametophyte ecology of these functional types (Watkins *et al.*, 2007a, b, c). Some epiphytic taxa have evolved fantastic degrees of desiccation tolerance in the gametophyte generation that likely contributes to establishment potential (Watkins *et al.*, 2007b) and ultimately controls species distributions. The gametophytes of epiphytic taxa also exhibit significant demographic differences from terrestrial species. Epiphytic gametophytes may live for years and perhaps decades and beyond, while their terrestrial counterparts have significantly reduced longevities (e.g. 1–2 months; Watkins *et al.*, 2007a). Such significant ecological differences do not disappear in the sporophyte generation and epiphytic taxa

exhibit divergent patterns of leaf-level nutrient and carbon relations relative to terrestrial taxa (Watkins *et al.*, 2007c).

What factors combine to influence the distribution of epiphytic and terrestrial species with such radical ecological differences? Most attempts to answer this question for epiphytes have focused on sporophyte ecology. Within canopy habitats, studies have demonstrated that water use efficiency and drought tolerance of sporophytes can affect epiphytic species distributions (Andrade and Nobel, 1997; Hietz and Briones, 1998). Epiphytic fern sporophytes also seem to be invested in biochemical (Hietz and Briones, 2001) and, to a more limited extent, morphological (Watkins *et al.*, 2006b) photoprotective measures which likely influence their distributions. Substrate preference also seems to play a significant role in structuring some species distributions (Moran *et al.*, 2003, Moran and Russell, 2004). Still others have attempted to quantify the effects of microclimate (Freiburg, 1998; Cardelús 2002; Cardelús and Chazdon, 2005), tree characteristics (Ter Steege and Cornelissen, 1989; Cardelús, 2002, 2007, Cardelús and Chazdon, 2005), and individual plant adaptations (Benzing, 1986, 1987) on overall epiphytic plant distributions.

In comparison to epiphytic species, our understanding of terrestrial species ecology is broader but remains limited for tropical species. Pioneering studies on the distributions of terrestrial ferns in the tropics have revealed the importance that edaphic specialization has on species distribution (Jones *et al.*, 2007, 2008; Tuomisto and Ruokolainen, 1994; Tuomisto and Dalberg, 1996; Tuomisto *et al.*, 1998; Tuomisto *et al.*, 2002). Sporophyte stress tolerance has also been demonstrated to influence the distribution of terrestrial tree fern species (Durand and Goldstein, 2001) and at the community level, both environmental and neighborhood effects have been shown to influence habitat specialization and act as an important determinant of the distribution of tree ferns (Jones *et al.*, 2007). While these studies have elucidated important aspects of fern ecology, few comparative studies on epiphytic and terrestrial species exist.

In a recent study comparing the distribution of epiphytic and terrestrial species along an elevational gradient, Watkins *et al.* (2006) found that out of 264 species only one grew as both a canopy epiphyte and a terrestrial species, and this from only a single site. A similar finding was reported by Kluge and Kessler (2006) along the same gradient in Costa Rica. Such a result is perhaps not surprising given the apparent ecological differences between these two groups. To understand better the patterns of fern habitat differentiation, we examined the vertical distribution of ferns on the trunk of an emergent canopy tree and compared this to species distribution in terrestrial plots. We asked the following questions: 1) How does overall species richness change and is there variation in the vertical distribution of epiphytic fern species along the trunks of an emergent tree species, 2) How does trunk fern richness and terrestrial fern richness compare and is there species overlap between habitats? 3) Are there differences in functional morphology between epiphytic and terrestrial species?

Materials and Methods

Species composition and distribution.—This study was conducted at La Selva Biological Station in Heredia Province, Costa Rica. The site is a 1400 ha tropical wet forest with an average rainfall of 4000 mm per year (McDade *et al.*, 1994). The trunks of six *Hyeronima alchorneoides* Allemao (Euphorbiaceae) trees were sampled for ferns using single rope climbing techniques (Perry, 1978). *Hyeronima alchorneoides* was chosen as this evergreen species maintains relatively high epiphyte species richness and has a well studied canopy habitat (Cardelús, 2002, 2005; Cardelús and Chazdon, 2005). All trees sampled were greater than 1 m in diameter above the buttresses with an average diameter among trees of 1.5 m. A 26 m transect, from the forest floor to the main bifurcation was established along the trunk of each of six trees and broken into contiguous 2 m × by 2 m plots. We found that 26 m was an ideal length that put the upper bound of the transect just below the first trunk bifurcation of all trees sampled. This small size 2 m × 2 m plots established along the trunk allowed for a finer level analysis of richness and cover. The average trunk area sampled per tree was 122 m^2. Identity, abundance (estimated by measurements of percent cover) frond morphology, and life form (e.g., primarily epiphytic, primarily hemi-epiphytic, and primarily terrestrial) were documented for each fern species in each plot. While the actual number of individuals in a given plot may be a better measure of species richness, accurate counts of individuals from six trees was difficult. Species such as *Hymenophyllum brevifrons* Kunze (Hymenophyllaceae) form mats of potentially hundreds of individuals; thus, we utilized percent cover as a proxy for dominance and ignored the actual number of individuals. Percent cover was estimated by determination of the total area covered in each 2 m × 2 m plot by a given species. As leaves can overlap, it was possible to a plot to have a total percent cover of >100% when summed across species.

For comparison of epiphytic species with hemi-epiphytic and terrestrial species, a circular plot with a radius of 26 m (total sampled area of 2122 m^2) was established terrestrially around the base of each sample tree. The 26 m radius plot was established to mimic the total tree height sampled. Each terrestrial plot completely encircled each sampled tree. The number of terrestrial individuals in these plots was often too low (4–5 individuals) to accurately allow for determination of percent cover; therefore, only presence/absence data was noted. Voucher specimens were collected from within each terrestrial plot and deposited in the National Herbarium of Costa Rica. For species identification we used the taxonomic concepts of Flora Mesoamericana (Moran *et al.*, 1995).

In addition to richness data, we quantified variation in leaf morphology among the different habitats sampled. Each species encountered was recorded as having compound or simple leaves. A chi square was run to determine if species from either habitat were associated with a given leaf morphology. We also evaluated specific leaf weight of species from three sections on the trunk. Data were collected from terrestrial plots, the buttress zone (0–2 m), and the

bifurcation zone (22–24 m). Due to limited time we choose these location subsets rather than sampling species along the entire trunk. The buttress zone and the bifurcation zone data also correspond to areas where we measured microclimate (see below).

Microclimate measurements.—Measurements of temperature and relative humidity were recorded on June 26, 2005. Three Hobo Pro Temperature/RH (Onset Corp., Bourne, ME, USA) data loggers were placed at three different locations on a single tree. One sensor was placed at 1.5 m above the forest floor to measure microclimate of the buttress zone, another sensor was suspended at 11 m above the forest floor to measure the mid-bole zone, and a final sensor at 23 m above the forest floor to represent the highest level or bifurcation zone. Temperature and humidity were recorded every 5 min for 12 hours. Water potential of the air was calculated using the formula: $\Psi = RT \ln e/e^0$, where R is the gas constant, T is the absolute temperature and e/e^0 is relative humidity expressed as a fraction (i.e. 50% r.h. = 0.5). This value was divided by the partial molal volume of water to convert to pressure units.

Light levels were measured with a Licor quantum sensor (Li-190, Lincoln, NE, USA) connected to a Licor data logger (Li-1400, Lincoln, NE, USA) at the same levels as temperature and humidity. Measurements were taken on June 27, 2005 and percent light transmittance was calculated by comparing sensor data to a control sensor measuring at the same time in an open field.

Results

Species composition and distribution.—A total of 40 fern species was found: 21 epiphytic species (plus gametophytes of Vittariaceae), 15 terrestrial species and 4 hemi-epiphytic species (Table 1). *Olfersia cervina* (Dryopteridaceae), was the only species found in both habitats (Table 1). The average number of epiphytic species found per tree was 11 (+/−2 species) with the sporophytes of *Vittaria stipitata* Kunze (Vittariaceae), *Elaphoglossum herminieri* (Bory & Fée) T.Moore (Elaphoglossaceae), *Oleandra articulata* (Sw.) C.Presl (Dryopteridaceae), and Vittariaceae gametophytes occurring on all individual trees (Table 1). Examination of presence/absence data suggests that terrestrial species are less abundant relative to the epiphytic species (Fig. 1). In addition, no single terrestrial species was represented in all 6 terrestrial plots. Only a single hemi-epiphytic species, *Polybotrya osmundacea* Humb. & Bonpl. ex Willd., was found in all six terrestrial transects.

Even though a significantly lower total trunk area was surveyed in the epiphytic when compared to terrestrial habitats, we encountered a higher diversity of epiphytic relative to terrestrial and hemi-epiphytic species (Table 1, Fig. 1). The number of epiphytic species remained relatively constant along the trunk up to 16 m, when diversity quickly increased (Fig. 1). Abundance (% cover) of epiphytes along the trunk did not follow this trend, but showed a strongly bimodal distribution (Fig. 2).

Microclimatic variation and extremes were differentially distributed over the trunk (Fig. 3). The buttress zone was consistently darker and exhibited

TABLE 1. Trunk and terrestrial plots where epiphytic, terrestrial, and hemiepiphytic fern species were collected. Shaded squares represent presence of a given species in a given plot. Circular terrestrial plots with a radius of 24 m were established at the base of each sampled tree. Hemiepiphytic species were all recorded from these terrestrial plots. Data from this study were gathered from La Selva Biological Station in Costa Rica.

	Plot Height on Tree Trunk (m)											
Epiphytic Species	0-2	2-4	4-6	6-8	8-10	10-12	12-14	14-16	16-18	18-20	20-22	22-24
Colchlidium serrulatum (Sw.) Bishop												x
Grammatid sp. 1												x
Microgramma lycopodioides (L.) Copel.												x
Phlebodium pseudoaureum (Cav.) Lellinger												
Elaphoglossum sp 2												
Microgramma reptans (Cav.) A.R. Sm.									x			
Elaphoglossum sp. 1									x			
Polypodium triseriale Sw.								x				x
Hymenophyllum brevifrons Kunze						x		x	x			x
Elaphoglossum herminieri (Bory ex Fei) T. Moor			x	x	x	x	x	x	x			x
Nephrolepis rivularis (Vahl) Mett. ex Krug.			x	x	x	x			x			x
Oleandra articulata (Sw.) Presl			x	x	x	x	x	x	x			x
Vittaria stipitata Kunze			x	x			x	x	x			x
Hecistopteris pumila (Spreng.) J. Sm.		x					x	x	x			x
Asplenium serra Langds. & Fischer	x	x			x			x	x			x
Hymenophyllum sp. 1	x	x	x	x	x	x		x	x			x
Elaphoglossum latifolium (Sw.) J. Sm.	x	x	x	x	x		x	x	x			x
Vittariaceae Gametophytes							x	x	x			x
Trichomanes godmanii Hook.	x	x	x	x								
Trichomanes ekmanii Wess. Boer	x	x	x									
Olfersia cervina (L.) Kunze	x											

	Plot Number					
Terrestrial Species	1	2	3	4	5	6
Dennstaedtia dissecta (Sw.) T. Moore			x		x	x
Adiantum obliqum Willd.	x	x				x
Alsophila cuspidata (Kunze) D.S.Conant	x	x				
Cyathea multiflora J. Sm.					x	x
Danaea wendlandii Reichenb. F.		x				x
Adiantum latifolium Lam.				x		
Blechnum occidentale L.						x
Diplazium macrophyllum Desv.						x
Pteris podophylla Sw.						x
Saccoloma inaequale (Kunze) Mett.	x					
Salpichlaena volubilis (Kaulf.) J. Sm.		x				
Tectaria dracontifolia (D.C. Eaton) Copel.				x		
Tectaria incisa Cav.			x			
Thelypteris curta (H. Christ) C.F. Reed						x
Thelypteris nicaraguensis (E. Fourn.) C.V. Morton		x				

	Plot Number					
Hemiepiphytic Species	1	2	3	4	5	6
Polybotrya osmundacea Humb. & Bonpl. ex Willd.	x	x	x	x	x	x
Olfersia cervina (L.) Kunze				x		x
Lomariopsis vestita E. Fourn.	x	x				
Polybotrya caudata Kunze	x					

significantly wetter air and less variation than the mid-trunk or bifurcation zone. Variation increased along the trunk with the most extreme and variable microclimate occurring in the bifurcation zone (Fig. 2).

There were also several species specific distribution patterns. For example, *Elaphoglossum sp.1* is a high light, high canopy species, whereas its congener *Elaphoglossum latifolium* (Sw.) J.Sm. seems to tolerate more variable microhabitats often occurring in the dark, wet buttress zone (Fig. 4). A similar pattern exists between *Hymenophyllum brevifrons*, a high canopy species, and the related *H. hirsutum* (L.) Sw. which follows a bimodal pattern similar to *E. latifolium*. In contrast, a pair of filmy ferns, *Trichomanes godmanii* Hook. ex Baker and *T. ekmanii* Wess.Boer are present at high densities on the low trunk and buttresses but neither occur in high canopy locations.

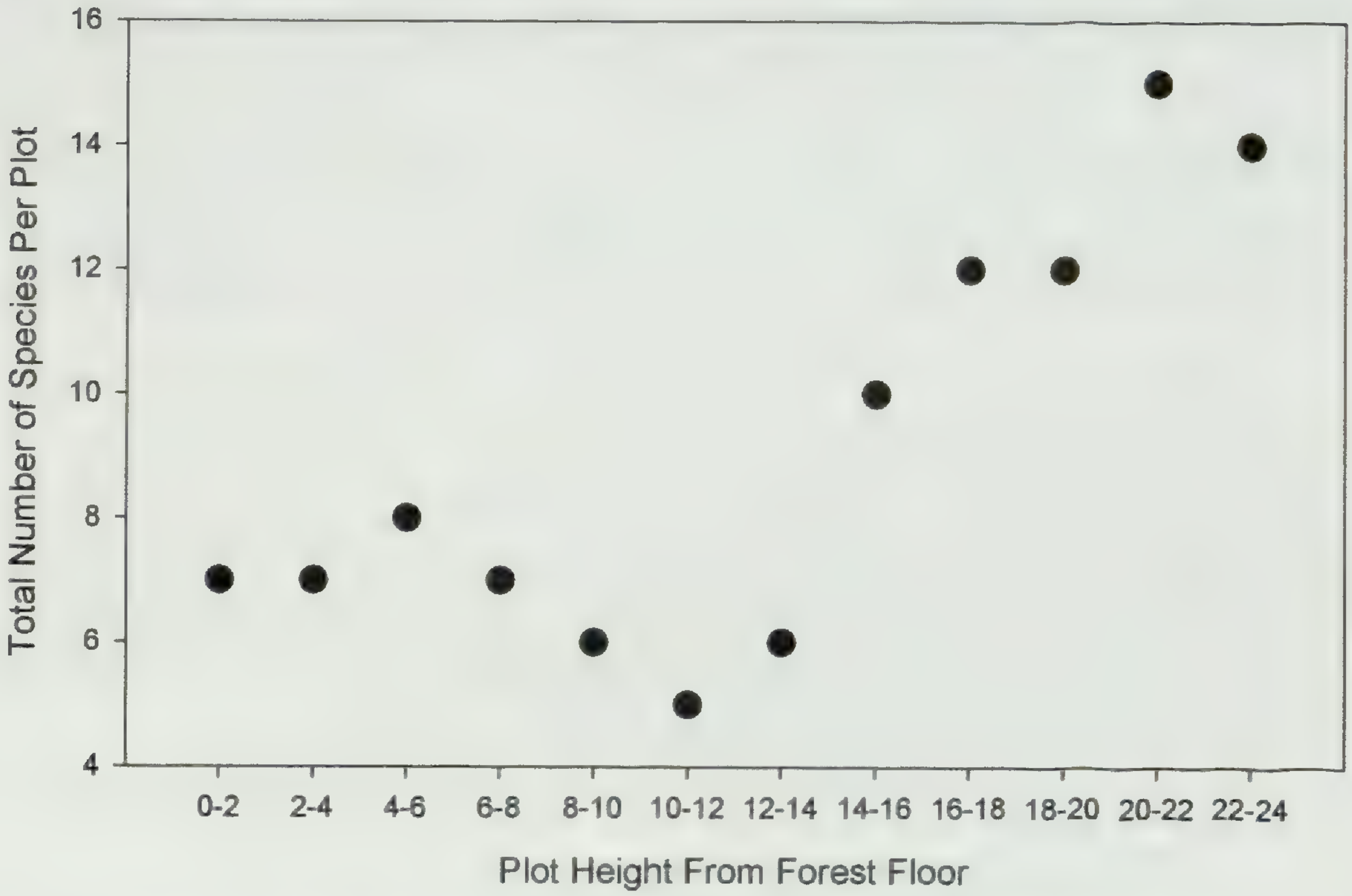

FIG. 1. Epiphytic, terrestrial, and hemiepiphytic fern species area curve sampled at La Selva Biological Station, Costa Rica. Epiphytic species were recorded on the trunks of six emergent canopy trees (*Hyeronima alchorneoides*) whereas terrestrial and hemiepiphytic species were collected in ground transects under each sampled tree.

Another intriguing distribution pattern unfolds when members of the Vittariaceae are examined. Due to their unique gemmae production (asexual propagules), we were able to identify the gametophytes of this family (Farrar, 1974). The gametophytes exhibit an interesting bimodal distribution occurring at both the buttress and the crown, while sporophytes of *Vittaria* were only encountered on high trunks (Fig. 5).

Frond Morphology.—When frond morphology was examined in epiphytic, terrestrial and hemi-epiphytic species, epiphytic ferns had significantly more species with simple leaves than terrestrial and hemi-epiphytic species ($\chi^2 = 18.13$; $p = 0.0001$) Thirteen of the 21 epiphytic species had simple leaves and there were no terrestrial or hemi-epiphytic species exhibiting this leaf morphology. Specific leaf weight also increased from terrestrial to bifurcation zone species (Fig. 6)

DISCUSSION

Species distributions.—In the first part of this study, our goal was to describe and compare the abundance (in terms of percent cover) and distribution patterns of epiphytic ferns in canopy habitats to determine if there is predictable vertical distribution of epiphytic fern species along the trunks of an emergent tree species. When the total number of species per plot was

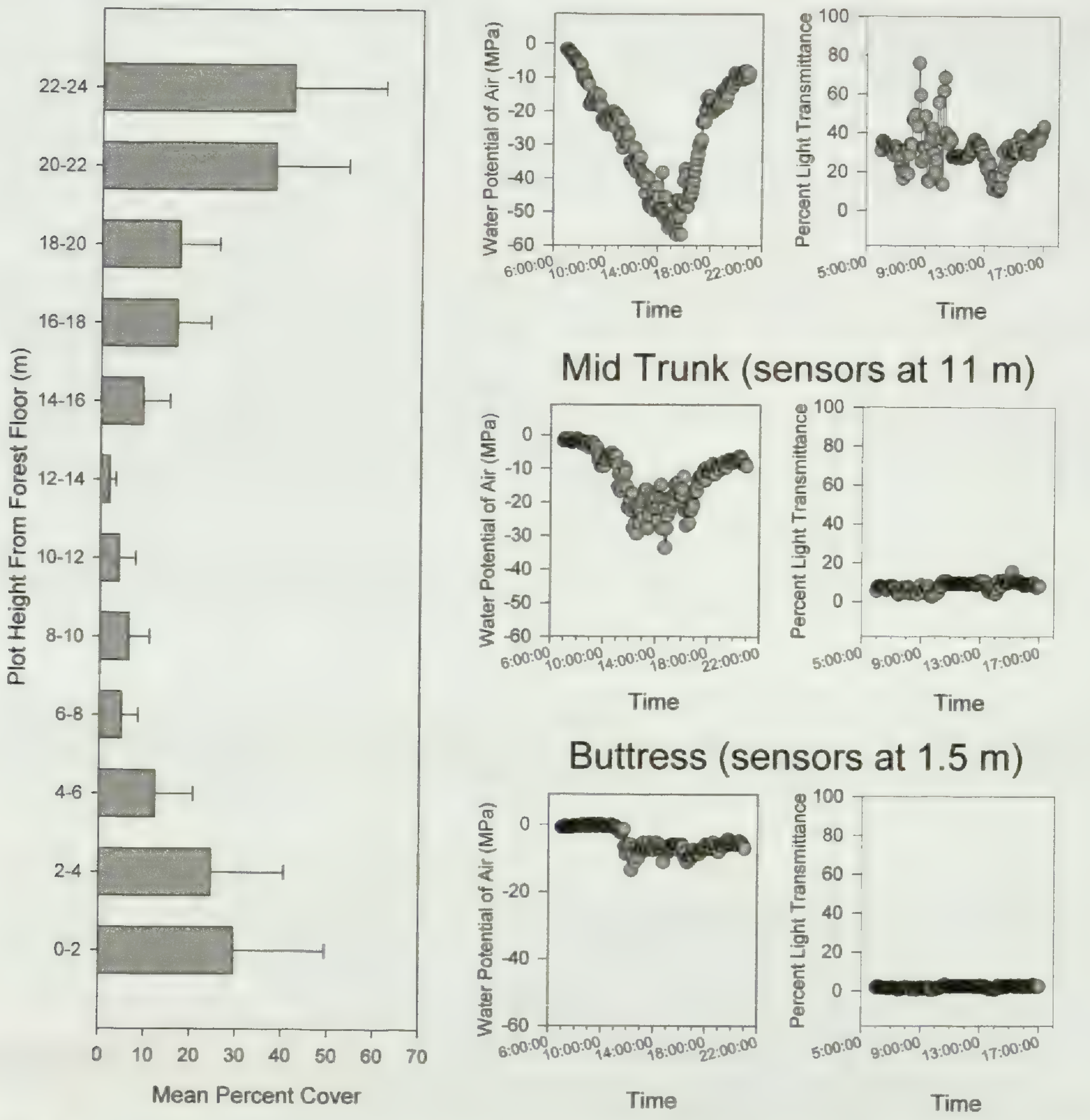

FIG. 2. The bimodal relationship of mean percent cover of all epiphytic fern species along sampled tree trunks. Humidity and light sensors were placed at three separate locations along the trunk: 1.5 m, 11 m, and 23 m. Microclimatic variables and light were measured at 5 min intervals at these locations.

examined, there was a predictable pattern of increasing species diversity with plot/tree height above 12 m (Fig. 3). The lower buttress zone (0–2 m) of any given tree was less diverse than the top bifurcation zone (22–24 m, Fig. 3). While diversity increased, percent cover exhibited a highly bimodal distribution (Fig. 2). Thus, while the number species increase with plot height, the total percent cover was similar between buttress and bifurcation zones. The buttress zone is homogenously dark and wet whereas the mid- and upper-trunk are brighter and drier and exhibit greater environmental

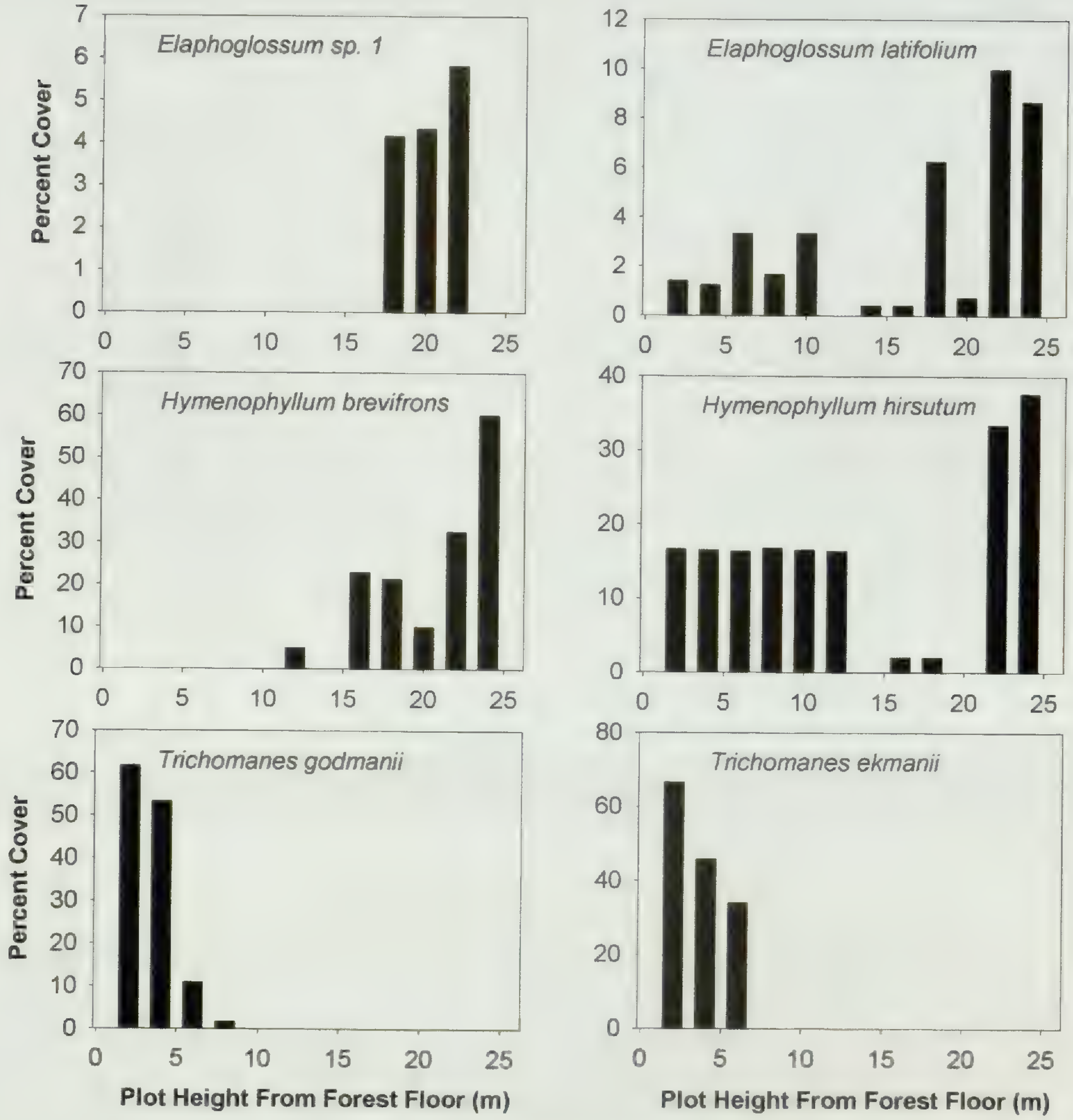

FIG. 3. The relationship of trunk height above ground on *Hyeronima alchorneoides* with the richness of epiphytic fern species. Highest richness was consistently within 2–3 m of the first branching of the trunk.

heterogeneity (Fig 2). These factors likely contribute to differences in diversity and abundance along the trunk. Indeed, whereas edaphic characters have been shown to influence terrestrial species diversity, light and water likely play an important role in shaping epiphytic species distribution (Hietz and Briones, 1998). Our data suggests that microenvironmental heterogeneity, rather than absolute values, is particularity important for epiphytic ferns.

Along with the zone specific microclimatic variation, we also found broad species specific patterns of distribution. The filmy ferns *Trichomanes ekmanii* and *T. godmanii*, dominated the dark buttress zone. The group, "filmy ferns," get their name from fronds that lack stomata, are one cell layer thick, and thus

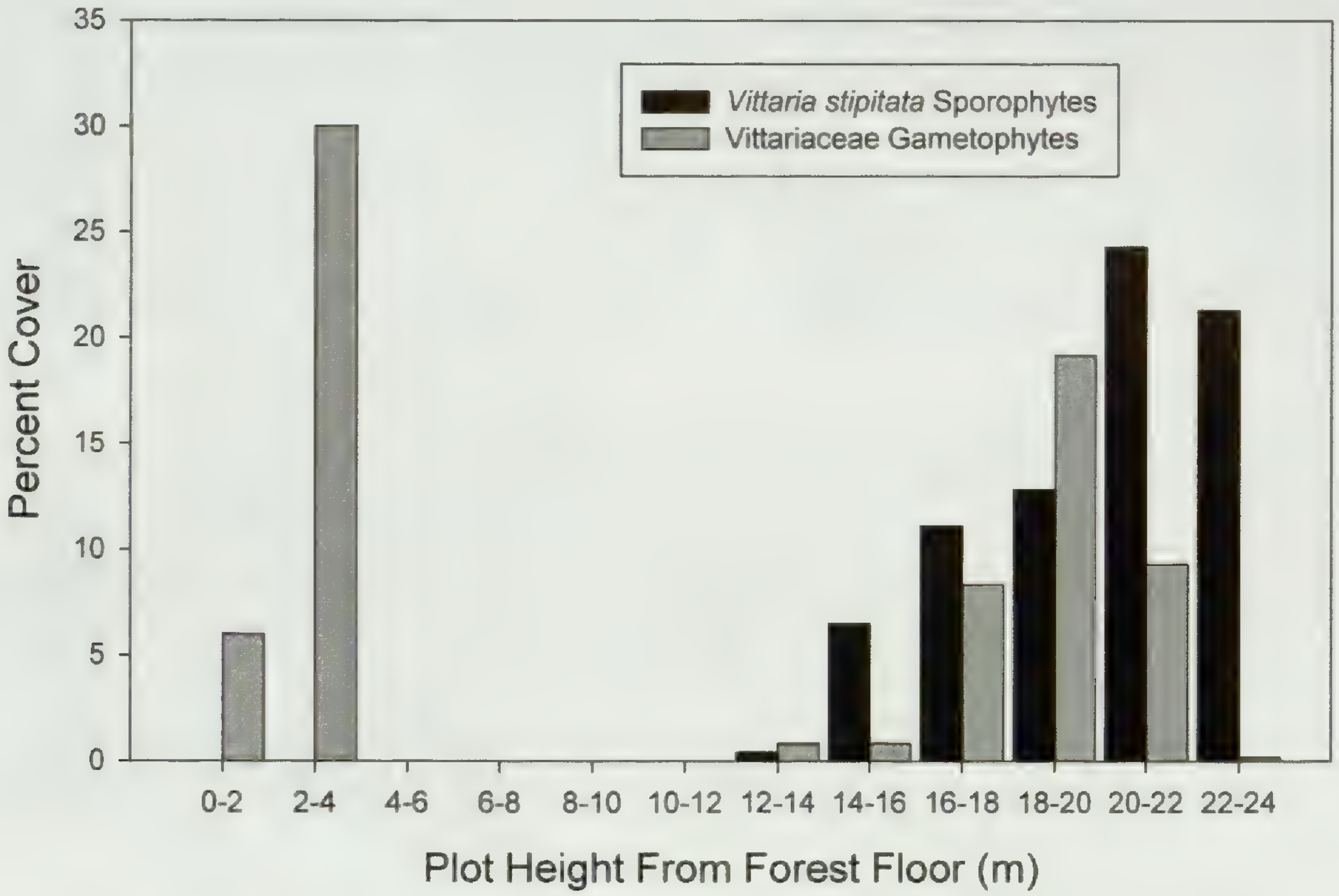

FIG. 4. Examples of a subset of epiphytic species distribution along the trunks of *Hyeronima alchorneoides*. Most species fell out into primarily buttress species or bifurcation species with few occurring across the trunk.

prone to desiccation. It is not uncommon to find large scale mortality of these two species following tree falls that expose them to brighter drier environments (pers. obs). *Hymenophyllum brevifrons* is similar in size to *T. ekmanii* and *T. godmanii* yet is completely absent from the dark buttress areas and quite abundant in high trunk habitats. *Hymenophyllum hirsutum* had a much broader range, occurring in most plots along the trunk (Fig. 4). The upper trunk had both a high percent cover and high species diversity which may reflect a more heterogeneous microenvironment to which different ferns are adapted. This variation may be important in maintaining high levels of fern diversity in tropical forests, especially on small local scales employed in this study.

An additional pattern to emerge from this study is the differential distribution of the gametophytes and sporophytes of the Vittariaceae. While the area remains poorly studied, it is thought that gametophytes may exhibit broader ecological distributions that their sporophyte counterparts (Sato and Sakai, 1980; Sato and Sakai, 1981; Peck *et al.*, 1990). Vittariod gametophytes are easily identifiable give there unusual morphology and gemmae production. We observed that Vittariod gametophytes exhibited a distinctly bimodal distribution relative to sporophytes which were confined to plots higher along the trunk (Fig. 5). There was not a single Vittariod sporophyte below 4 m on any of the trees sampled. We encountered hundreds of gametophytes from potentially several different non-Vittariod species which suggests that the

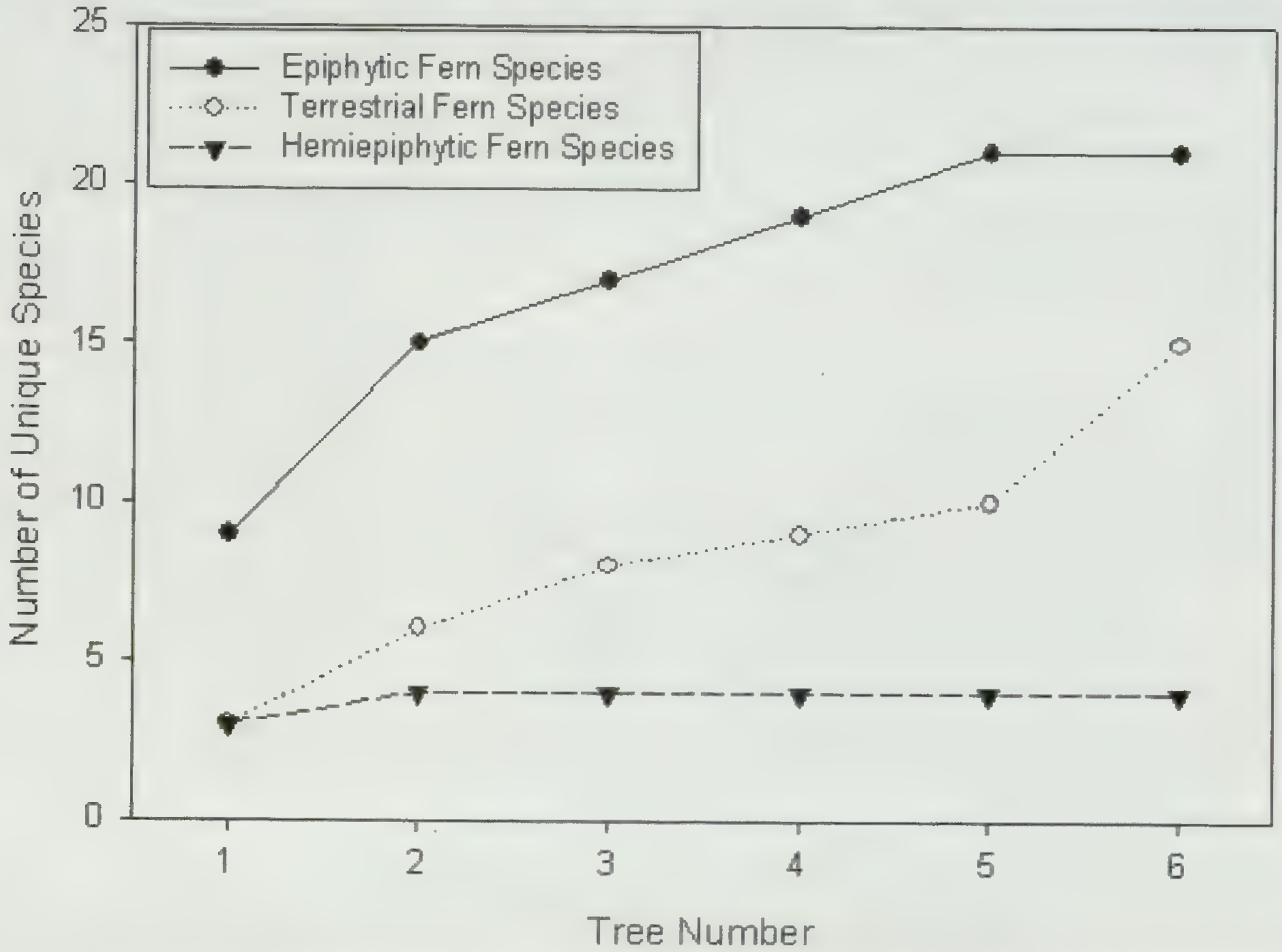

FIG. 5. Distribution of sporophytes and gametophytes of the Vittariaceae. Sporophytes are only found on the upper portions of the trunk, whereas gametophytes were found in both the upper portions and lower portions of the trunk.

gametophytes of other species may exhibit similar patterns of distribution. Gametophytes may be more highly adapted to growth in dark environments as the carbon budget of an individual, and thus growth rates, are small compared to the needs of the sporophyte (Farrar, 1998). Whereas gametophytes can establish in a broader range of environments, sporophytes may be more restricted to more stable niches. Greater ecological plasticity in the gametophyte generation may be important in "habitat exploration" for species as it is the first living stage to encounter new environments. Data from temperate species has shown that gametophyte plasticity is important to establishment and sporophyte distributions (Greer *et al.*, 1997).

Species diversity.—The second part of this study examined how trunk fern diversity and terrestrial fern diversity compare and asked if there is species overlap between habitats. The area sampled on all six trees was less than the area sampled in the first terrestrial plot, yet the number of epiphytic species is much higher than terrestrial species (Fig. 1). As with any study on tropical species diversity, our species area curve indicates that we under-sampled terrestrial species. We ceased to discover any additional epiphytic species, yet we know from an earlier floristic survey of La Selva (Grayum and Churchill, 1987) that this too represents an underestimation of epiphytic and hemi-epiphytic species.

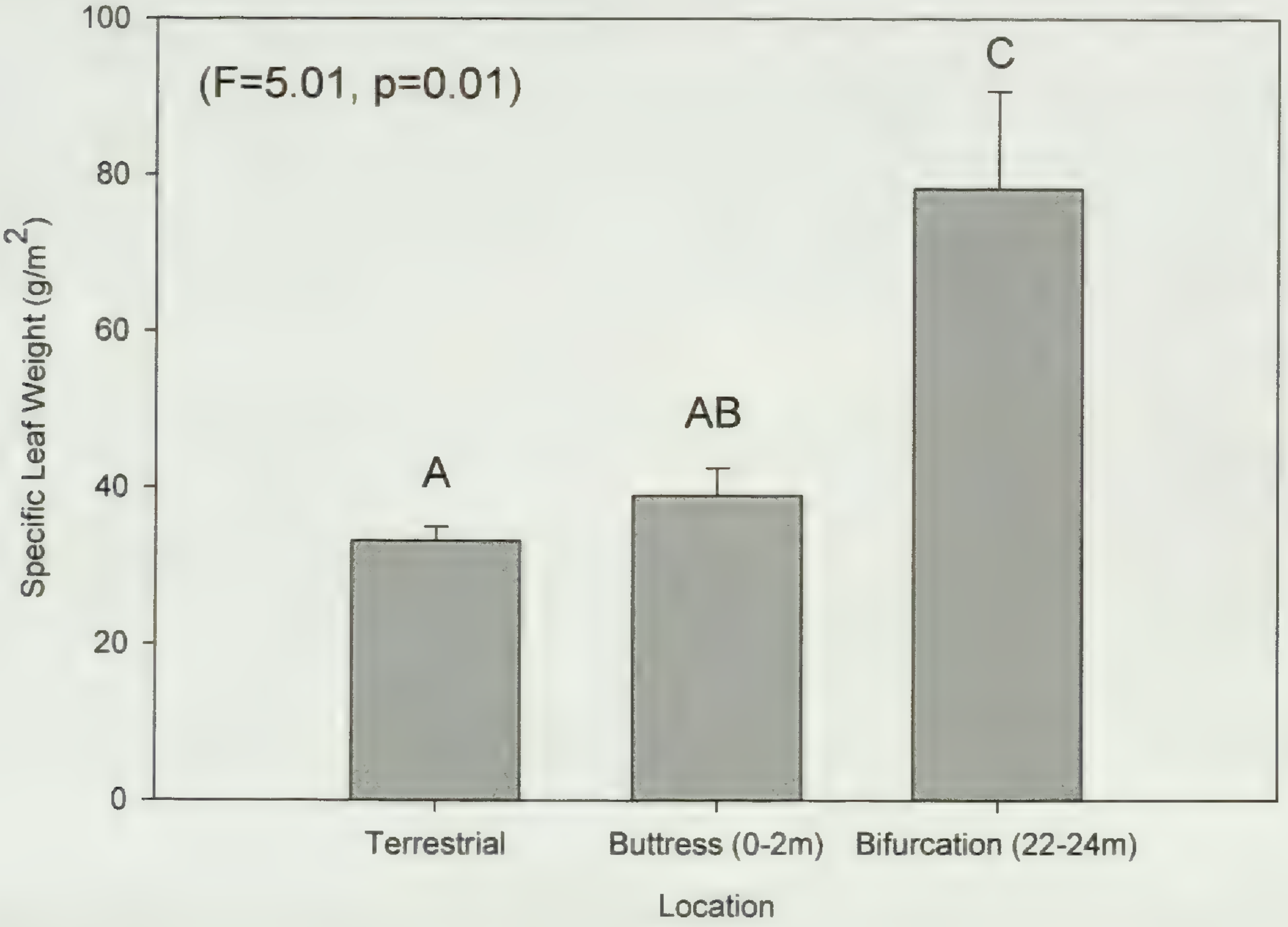

FIG. 6. Specific leaf weight of terrestrial, buttress epiphytes, and bifurcation epiphytes growing on *Hyeronima alchorneoides* at La Selva Biological Station, Costa Rica.

Nevertheless, in this study, epiphytic species were more diverse than terrestrial and hemi-epiphytic species. While it is has been difficult to show that host specificity influences epiphyte composition (Zotz and Vollrath, 2003) it is known that certain tree species harbor greater diversity and numbers of epiphytes (Cardelús, 2002). The tree chosen for this study has a diverse and abundant epiphyte flora relative to other emergent tree species in La Selva.

The epiphytic fern flora of *Hyeronima alchorneoides* makes for interesting comparisons with the terrestrial fern flora as the species grows on both alluvial bottoms and upland terraces. We were thus able to sample a diversity of soil types and found that alluvial bottoms were areas of particularly high terrestrial fern diversity. For example, the final terrestrial transect sampled happened to occur along a small stream on one of the alluvial bottoms at La Selva. The number of terrestrial species in this particular plot was almost double that of the most diverse terrestrial plot in the sample. While other factors may be involved, this observation further supports the importance of edaphic factors to patterns of terrestrial fern distribution (Tuomisto and Ruokolainen, 1994; Tuomisto and Poulsen, 1996; Tuomisto *et al.*, 1998).

When we examined the species overlap between habitats, we found that *Olfersia cervina* was the only species that was found growing in epiphytic and terrestrial habitats. This species has been described as a low climber (Moran, 1995) and as a hemi-epiphyte which could exclude it from both the epiphytic

and terrestrial groupings. The species was only observed in pockets of deep soil that form on buttresses and never above 2 m from the forest floor. This is an unusual species in that it is most frequently encountered growing on large fallen trees in advanced stages of decay (pers. obs). *Hyeronima* often forms buttresses that can collect large amounts of detritus and thus provides an important habitat for *Olfersia*. Perhaps of greater interest is that there was not a single species that grew on terrestrial soil and on the upper trunk further corroborating the reports by Watkins *et al.* (2006). One question that has often plagued pteridologists is how the evolution of epiphytisim actually came about. In spite of the fern's dispersal syndrome, such intensive local sampling effort combined with regional studies (Watkins *et al.*, 2006) suggest that reciprocal establishment of epiphytic and terrestrial species is rare. In the ferns, epiphytisim likely arose through some intermediate form, most likely passing through some hemi-epiphytic form before radiation into a completely epiphytic condition.

Leaf morphology.—There are striking differences in leaf morphology between the epiphytic and terrestrial species studied here. The majority of epiphytic species (13 of 21) have simple leaves whereas terrestrial and hemi-epiphytic species have compound morphologies. Interestingly, of the epiphytic species with compound leaves, only two species in the Hymenophyllaceae had leaves that were more than once pinnate. In an opposite pattern, 11 of the 15 terrestrial species exhibited leaves that were more than once divided; the other four species had once pinnate leaves. These patterns are repeated throughout the Costa Rican pteridoflora (pers. obs) and this convergence of leaf form in the canopy, in several divergent lineages, suggests that these traits are adaptive and are under direct selective pressure. Epiphytic species from the bifurcation zone also had significantly increased specific leaf weight compared to terrestrial and buttress epiphytes. Canopy habitats tend to be hotter, drier (Fig. 2), and experience more wind than terrestrial habitats in most tropical forests. Thus, it is likely that the combination of both energy and mechanical aspects have influenced the evolution of leaf morphology and leaf thickness in epiphytes.

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Eukaryotic Microbial Communities Associated with the Rhizosphere of the Temperate Fern *Thelypteris noveboracensis* (L.) Nieuwl.

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Abstract.—Microbial communities, associated with terrestrial mosses (Bryopsida) and the rhizosphere of agricultural and natural occurring seed plants, have been rather extensively examined; but less is known about associations with seedless vascular plants, including ferns. The New York fern (*Thelypteris noveboracensis*), typically found within deciduous forests, occurs in locally extensive stands in North America extending from northeastern Canada to southeastern U.S.A. Soil samples were obtained in autumn (2007) and early summer (2008) within a plot of *T. noveboracensis* in the understory of deciduous trees in the forest reserve at Torrey Cliff, NY to document the rhizosphere (root-associated) density of commonly occurring heterotrophic eukaryotic microbes (protozoa), including microflagellates, naked amoebae and testate amoebae. The ranges in densities (number/g soil dry weight) are as follows: microflagellates (6.5×10^6–1.3×10^8), naked amoebae (1.8×10^3–4.0×10^6) and testate amoebae (ca. 400). Very few active ciliates were observed. This is the first report of microbial communities associated with the rhizosphere of ferns and provides a step toward a more complete documentation of protozoa associated with plant communities. Some comparative data of protozoa associated with mosses and seed plants are also presented.

Key Words.—microbial diversity, microflagellates, naked amoebae, New York fern, soil biology, terrestrial ecology, testate amoebae

In recent decades, considerable ecological research has focused on understanding the coupling of aboveground and belowground processes; that is, how primary production of plant organic matter, including exudates from roots, affects the belowground soil microbial communities. Bacteria supported in part by organic matter from plants, and stimulated by the presence of eukaryotic microbes (protozoa) and secondarily by bactivorous nematodes, mineralize and recycle soil nutrients, thus enhancing plant growth. Therefore, understanding the functioning of land plants must include knowledge of the protozoan community in soils and the rhizospheres surrounding roots. Soil microbial communities associated with terrestrial mosses (e.g., Anderson, 2006, 2008) and the rhizosphere of agricultural and naturally occurring seed plants, have been extensively studied in a wide range of terrestrial locations (Bamforth, 1984; Clarholm, 1985, 1989; Foissner, 1987; Griffiths 1990; Cowling, 1994; Anderson, 2000; Adl, 2003; Li *et al.*, 2005). However, less is known about microbial communities associated with the rhizosphere of seedless vascular plants, including ferns; although, they are widely distributed geographically from arctic to tropical habitats (e.g., Moran, 2004). Fern rhizomes and roots, in addition to decaying shed fronds, are substantial

sources of organic matter that may support rich microbial communities. Fern root systems also may secrete possible allelopathic and antimicrobial substances (e.g., Horsley, 1977; Stetsenko *et al.*, 1984; Hill and Silander, 2001). Hence, a better understanding of microbial communities associated with fern rhizospheres is warranted to more fully document the microbial communities associated with a wide range of plant groups, and eventually to more completely understand the dynamics of the interactions of ferns with associated terrestrial eukaryotic microbes. The purpose of this research was to contribute to our knowledge of fern rhizosphere microbial communities by examining the eukaryotic microbes associated with the rhizosphere of *Thelypteris noveboracensis* (L.) Nieuwl., a widely distributed, temperate fern in eastern Canada and U.S.A.

Materials and Methods

Sample site.—Soil samples, using a LaMotte model EP corer, were obtained from the rhizosphere of a well-established plot of *T. noveboracensis* in the Torrey Cliff Forest Reserve at Palisades, NY (40° 59′ 58″ N; 73° 54′ 30″ W). Three separate soil cores approximately 2 cm long were taken on each sampling date and combined to obtain a more representative soil sample. The mixed sample was placed in a sealed plastic bag and immediately returned to the Lamont-Doherty Observatory laboratory for analysis. Samples were taken in October and November of 2007 and early June of 2008 to provide some evidence of seasonal differences. *Thelypteris noveboracensis* grows by a spreading rhizome and each plant produces an extensive patch of growth. For example, at Black Rock Forest (Cornwall, NY), an expansive patch occupying ca. 0.1 km^2 (10 ha) developed in a forest clearing within several seasons after opening of the canopy (Schuster, pers. comm., 2008).

Soil analyses.—Moisture content (expressed as % w/w) was determined gravimetrically by difference in weight between the fresh sample and after drying to constant weight at 109 C. The pH of the soil samples in aqueous suspension (5 g per 50 ml distilled water) was obtained using an Accumet™ model 15 pH meter (Fisher Scientific, Fairlawn, NJ). Percent (w/w) organic content was determined by difference in weight between the fresh sample and after combustion at 375°C for 12–16 hours.

Microbiota.—Densities of bacteria that typically serve as prey for the eukaryotic microbiota were estimated by direct fluorescent microscopic counting (Anderson *et al.*, 2001). Microflagellate densities in aqueous extracts of the soil samples, fixed in 2% TEM grade glutaraldehyde, were determined using an acridine orange fluorescence method (Anderson *et al.*, 2001) adapted from Hobbie *et al.*, 1977. Testate amoebae and ciliates were enumerated by exhaustive examination of 3 ml (50 µl subsamples per observation) of aqueous-suspended soil samples fixed with 2% TEM grade glutaraldehyde and stained with Lugol's iodine (Anderson, 2008). Densities of naked amoebae were estimated using a standard culture observation method (COM), and cyst densities were estimated using the dried aliquot culture observation method

(DCOM) as published previously (Anderson, 2000). For the COM, a freshly collected sample of soil was suspended in micropore-filtered pond water (MFPW) in a ratio of 1 g in 50 to 80 ml total, and thoroughly dispersed. A 10 µl aliquot was dispensed per well of a 24-well Falcon™ tissue culture dish containing 2 ml of MFPW and a small cube of malt yeast agar (MYA) serving as a nutrient source for prey bacteria. Triplicate plates were prepared for each sample. After 10 to 14 days incubation at 25°C, each well was examined to determine the presence or absence of a given amoeba morphospecies indicating, if present, that at least one individual of that morphospecies was in the 10 µl aliquot. The total tally of each morphospecies was obtained and converted to number per ml of the original suspension. The number of amoebae per g dry weight of soil was calculated based on the weight of soil used to make the original suspension (Anderson, 2000). For the DCOM method, a 10 µl aliquot of the soil suspension was deposited in each of the dry wells of the Falcon culture dish and rapidly dried by flowing air before the MYA and 2 ml of MFPW were added. Thus, only the encysted amoebae survive the drying; and their count, based on observations of emergent morphospecies in the wells of the tissue culture dish, indicates the density of encysted amoebae in the original soil sample. Diversity of naked amoebae morphospecies was determined using the Shannon-Wiener formula ($H = 1 - \Sigma\ p_i \cdot \log_2 p_i$.) where p_i is the proportion of each morphospecies relative to the total. All microscopic observations were made with a Nikon Diaphot™ inverted compound microscope using phase contrast optics.

Results

Fern soil moisture at the sampling site ranged from 30 to 34%, pH was 4.4, and the organic content of the soil was 20%. Densities (number/g dry weight) of bacteria ranged from 2.5 to 7.1×10^9 comparable to published data for other terrestrial sites. The densities of protozoa in the autumn and June samples are presented in Table 1, including the percentages (in parentheses) of naked amoebae that were encysted and of testate amoebae that were atrophied or present as empty shells. No ciliates were observed in the Lugol's iodine preserved samples, which is consistent with other reports that a significant number of soil ciliates are usually encysted under typical field conditions, except when soil water is elevated following heavy precipitation (e.g., Foissner, 1987). However, as observed with terrestrial moss samples (Anderson, 2008), occasional ciliates were observed in the COM culture wells, confirming that encysted ciliates were probably present and became active when more fully hydrated. The diversity of naked amoebae was relatively high (H = ca. 3.0). The major genera of naked amoebae identified included *Acanthamoeba*, *Arachnula*, *Cochliopodium*, *Hartmannella*, *Mayorella*, *Saccamoeba*, *Thecamoeba*, *Vahlkampfia*, *Vexillifera* and *Vannella*, further indicating relatively rich species diversity. The major genera of testate amoebae were *Trinema*, *Euglypha*, *Tracheleuglypha*, *Corythion* and occasionally *cryptodif-*

TABLE 1. Densities of microbiota in fern soil samples during autumn 2007 and June 2008.

Sample date	Micro-flagellates	Naked amoebae	Testate amoebae
Oct. 24	6.5×10^6	4.0×10^6 (53%)	400 (66%)
Nov. 8	9.8×10^6	2.1×10^6 (48%)	444 (80%)
June 7	1.3×10^8	1.8×10^3 (50%)	400 (20%)

Densities are number/g dry weight of soil. All microflagellates counted appeared to be trophic stages; the percentages of encysted naked amoebae and of testate amoebae with empty shells or atrophied cytoplasm are presented in parentheses.

flugia. A globose testate amoeba, possibly *Geopyxella*, was occasionally abundant.

Interestingly in this study, the density of naked amoebae in the June sample (ca. 2×10^3) was less than in the October and November samples (ca. 4.0 and 2.0×10^6, respectively). However, the microflagellate density was substantially higher in June (ca. 1×10^8) compared to the October and November samples (6.5 and 9.8×10^6, respectively). While the total density of testate amoebae was relatively constant for the three sampling dates (ca. 400), the percent inactive was lowest in June (20%). Comparative data with other plant groups obtained from some representative published sources, including terrestrial mosses and the rhizosphere of seed plants, are presented in Table 2. In general, the densities of flagellates and naked amoebae were within the broad range found in other plant communities, but the testate amoeba densities were somewhat lower.

DISCUSSION

This research presents some of the first data on eukaryotic microbial organisms associated with the rhizosphere of a temperate fern in a northeastern U.S.A. hardwood forest. The robust growth of the fern's creeping rhizome, and production of organic matter due to secretory products, exfoliation of rhizome scales, deposition of shed fronds, etc., may contribute to the relative high organic content of the rhizosphere soil (20%) and resulting relatively high moisture content (ca. 30%), thus supporting a robust microbial community. Overall, the densities of fern-associated protozoa are reasonably similar to that found in other plant communities, but as discussed below in some cases they exceeded the densities associated with seed plants in organically enriched soils. The published data on seed plant rhizosphere microbiota encompasses a broad range of habitats including deserts, cultivated soils, grasslands, temperate forests, and tropical rain forests (Table 2). A more informed analysis is possible when the data from the current study are compared to some illustrative published results from organically rich soils. For example, in organically rich soils, the reported density of flagellates was in the range of 10^5, and naked amoebae 1.4 to 2.6×10^4 (Griffiths, 1990), or as much as 2×10^5 in tropical soil litter (Bamforth, 2006). Clarholm (1994) reported peak flagellate densities of ca. 4×10^6 in litter of a pine forest following rain

TABLE 2. Densities of microbiota in the fern rhizosphere compared to some published data for other plant communities.

Plant group	Terrestrial Mosses[1]	Fern *T. noveboracensis*	Seed plants[3]
Flagellates	1×10^5–4×10^7 (Anderson, 2008)	1×10^7–1×10^8	1.3×10^5–4×10^6 (Griffiths, 1990; Clarholm, 1994)
Naked amoebae	3.5×10^3–3.6×10^4 (Anderson, 2006, 2008)	2×10^3–4×10^6	2×10^3–2×10^6 (Clarholm, 1989, 1994; Griffiths, 1990; Bamforth, 2006)
Testate amoebae	3×10^2–6×10^3 (Anderson, 2008)	90–300[2]	1.3×10^4–3.7×10^5 (Lousier, 1982; Wanner and Xylander, 2005; Bamforth, 2006)

[1] Data are from published sources on terrestrial mosses in Torrey Cliff, NY and Toolik, AK.
[2] Adjusted data for living individuals, excluding those atrophied or with empty shells.
[3] Data for seed plants are largely from cultivated soils and tree-bearing sites.

during September 1977, but the lowest values were on the order of 2×10^6. Both the flagellates and naked amoebae in the fern soil examined in this study reached densities substantially higher, including published data for terrestrial mosses (Anderson, 2006, 2008). The densities and diversity of testate amoebae are less than those reported in other terrestrial sites, although the genera detected are not particularly unusual for natural soils (Foissner, 1987; Cowling, 1994).

Overall, this study indicates that *T. noveboracensis*, at least at the Torrey Cliff site, sustains a rich and substantial community of soil microbiota. Additional research is needed to replicate this work at other geographic locations, including other fern species, to more fully document fern rhizosphere microbiota. However, these data provide at least the first evidence of protozoan densities in a fern rhizosphere at a temperate woodland site. In general, there is limited biogeographic data on terrestrial microbial communities associated with diverse plants and much additional systematic research is needed to more fully understand the population structure and ecological dynamics of plant-associated microbial communities on a global scale.

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Structure and Organization of the Rhizome Vascular System of Four *Polypodium* Species

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ABSTRACT.—The present investigation is a detailed study of the vasculature of the rhizome of four species of *Polypodium* (*P. cambricum*, *P. fauriei*, *P. interjectum*, and *P. sibricum*). The vascular architecture of the rhizomes of the *Polypodium* species studied denotes a line of reduction and simplification of characters. The characteristic nature of the association of branches with leaves in the species of *Polypodium* studied seems to be significant. Vascular morphology of the rhizome of *Polypodium* does not support a close relationship with *Goniophlebium* as has previously been hypothesized. However, more extensive study of *Polypodium* is needed to arrive at any definite conclusion.

KEY WORDS.—Polypodiaceae, rhizome, vascular system, *Polypodium*

The genus *Polypodium* is typified by *P. vulgare* Linn., a fern of the north temperate zone of the world. *Goniophlebium* is regarded by some taxonomists as congeneric with *Polypodium* while many prefer to regard it as separate genus (Rodl-Linder, 1990; Schneider *et al.*, 2004; Srivastava and Khare, 2005). Though predominantly a neotropical genus, *Polypodium* includes a few species in Africa and eastern Asia and one, *P. vulgare* Linn., the type species, in Europe.

Christensen (1938) redefined the genus *Polypodium*, excluding hundreds of species treated in it by earlier authors. At the same time he included *Goniophlebium* in the genus *Polypodium*, which he considered a natural genus of about 50 species in tropical and subtropical America, Europe and Asia to Polynesia. Ching (1940) merged *Goniophlebium* into *Polypodium* which he placed in tribe Polypodieae of the subfamily Polypodioideae. Holttum (1949), who recognized five groups within the Polypodiaceae, included *Goniophlebium* in *Polypodium*, which he placed in Phymatodes group. Copeland (1947) and de la Sota (1973) preferred to separate the palaeotropic species to constitute the genus *Goniophlebium*, which they regarded as closely related to *Polypodium s.s.* Nayar (1970, 1974) and Crabbe *et al.* (1975) included *Polypodium* and *Goniophlebium* in their subfamily Polypodioideae of the family Polypodiaceae. Pichi-Sermolli (1977) recognized 14 groups within the Polypodiaceae, one of them included the genera

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Polypodium and *Goniophlebium*. Tryon and Tryon (1982) and Rodl-Linder (1990) considered *Goniophlebium* an Old World genus with articulate pinnae and not closely related to *Polypodium* s.s., which is predominantly a New World genus. Hennipman *et al.* (1990) provisionally included *Goniophlebium* in *Polypodium* sensu lato, tribe Polypodieae of the subfamily Polypodioideae. Recently, Smith *et al.* (2006) included both in Polypodiaceae of the order Polypdiales under Polypodiopsida.

In the genus *Polypodium*, rhizomes are short to long creeping, the rhizome scales are peltate or pseudopeltate rarely basifixed and clathrate or opaque with a broad central band. Fronds are uniform, monomorphic and simple with the lamina usually pinnatifid or pinnate and with no laminar hairs or scales. Vascular morphology of the rhizome of ferns is currently well accepted as a conserved feature, which is minimally affected by the environment and is a highly reliable comparative criterion thought to be of significance in taxonomic and phyletic studies of homosporous ferns. Hence, the structure and organization of the vascular system in the fern shoot have been useful in comparative studies (Tansley, 1907–08; Bower, 1910, 1914, 1915, 1917, and 1918; Hayata, 1927, 1928; Tardieu-Blot, 1932; Ogura, 1972; Ching, 1940; Holttum, 1964; Nayar and Chandra, 1967; Nayar *et al.*, 1968). In recent years, pteridologists have also demonstrated the importance of the vasculature of the rhizome in phylogeny (Kato, 1972; Lucansky and White, 1974; Chandra and Nayar, 1975; Chandra and Kaur, 1976; Chandra, 1982; Chandra *et al.*, 2003; Hovenkamp, 1990; and Srivastava *et al.*, 2007).

Even with a long history of vasculature research, our knowledge of the vascular system of the rhizome of the genus *Polypodium* is meager, and, to date the only detailed description of the vasculature of the rhizome of *Polypodium vulgare* was provided by Srivastava and Khare (2005). The structure and organization of vascular system in the rhizome of most species of *Polypodium* remain almost unknown, except for few anatomical observations on the rhizome of *P. microrhizoma* Clarke ex Baker and petiole of *P. amoenum* Mett. (Bir and Trikha, 1980), and gross morphological details of *Polypodium formosanum* Baker and *P. glaucophyllum* Kunze (Hovenkamp, 1990).

The present investigation is a detailed study of the vasculature of the rhizome of four species of *Polypodium* (*P. cambricum* L., *P. fauriei* Christ, *P. interjectum* Shivas and *P. sibricum* Sipliy) with more emphasis on the arrangement of leaf gaps, number and departure of the leaf trace strands, association between leaf gap/branch gap/leaf trace/branch trace, and association of mechanical tissue. Our goal is to assess the potential value of these characteristics in taxonomic and phyletic considerations.

Materials and Methods

For the present study, four species of *Polypodium* were obtained from Russia through the courtesy of Dr. Nina M. Derzhavina, Herbarium, Orel State University, Russia. They are *P. cambricum* L. (*N.M.Derzhavina 87*, OHHI), *P.*

fauriei Christ (*N.M.Derzhavina 10*, OHHI), *P. interjectum* Shivas (*N.M.Derzhavina 94*, OHHI) and *P. sibricum* Sipl. (*N.M.Derzhavina 15*, OHHI).

Vasculature morphology was studied mainly from serial sections from hand as well as microtome sections (cut at 100–130 μm) of adult rhizomes fixed in F.A.A. and stored in 70% ethyl alcohol. Anatomical observations recorded here are based on microtome sections stained with safranin and fast green. Stelar organization was studied from three-dimensional reconstructions based on camera lucida tracings of the outline of the vascular strands in serial sections. Because of the significance of vascular organization in the rhizome of ferns in taxonomic and phyletic considerations, particular attention was paid to the general form and shape of the vascular cylinder, leaf gap, leaf trace, branch gap, branch trace and the association of the branch with the leaf.

Results

Rhizomes are moderately stout (*P. cambricum* and *P. interjectum*) to soft and slender (*P. fauriei*, and *P. sibricum*) bearing leaves restricted to two alternating, dorsal rows. The diameters of the rhizome ranges from ca. 2 mm to ca. 8 mm. Branches are usually associated with leaves on the abaxial side away from the dorsal median plane of the rhizome.

Transverse section of the rhizome in any plane usually shows 9–12 cylindrical to sub-cylindrical vascular bundles, which are distributed roughly in a circle in the ground tissue. The rhizome is soft, parenchymatous with the ground tissue uniform (not differentiated into cortex and pith) and having dense starch deposits. Sclerenchyma strands, as found in other polypodiaceous ferns, are absent in the species of *Polypodium* studied here. The epidermis is single layered and consists of regularly arranged small, thin-walled, rectangular cells.

Form of the Vascular Cylinder

The vascular cylinder of the rhizome is basically similar to the common types in the Polypodiaceae and is a highly perforated dictyostele with much elongated lacunae forming a conspicuous loose reticulum (Figs. 1–4). It is pierced with two alternating, large overlapping leaf gaps on the dorsal side and many large lacunae elsewhere so that the vascular strands are slender and cylindrical. In all four species the vascular cylinder is dorsiventral with leaf gaps closely placed at the dorsal surface so that the successive ones of the two rows overlap conspicuously. The area of the vascular cylinder between the two rows of the leaf gaps is slightly thicker than others constituting a distinct dorsal median vascular strand.

Root traces are restricted to the ventral half of the vascular cylinder and originate as superficial, solitary vascular strands from the outer surface of the stelar cylinder. As the root traces passes through the cortex of the rhizome it acquires a thick sheath of sclerenchyma.

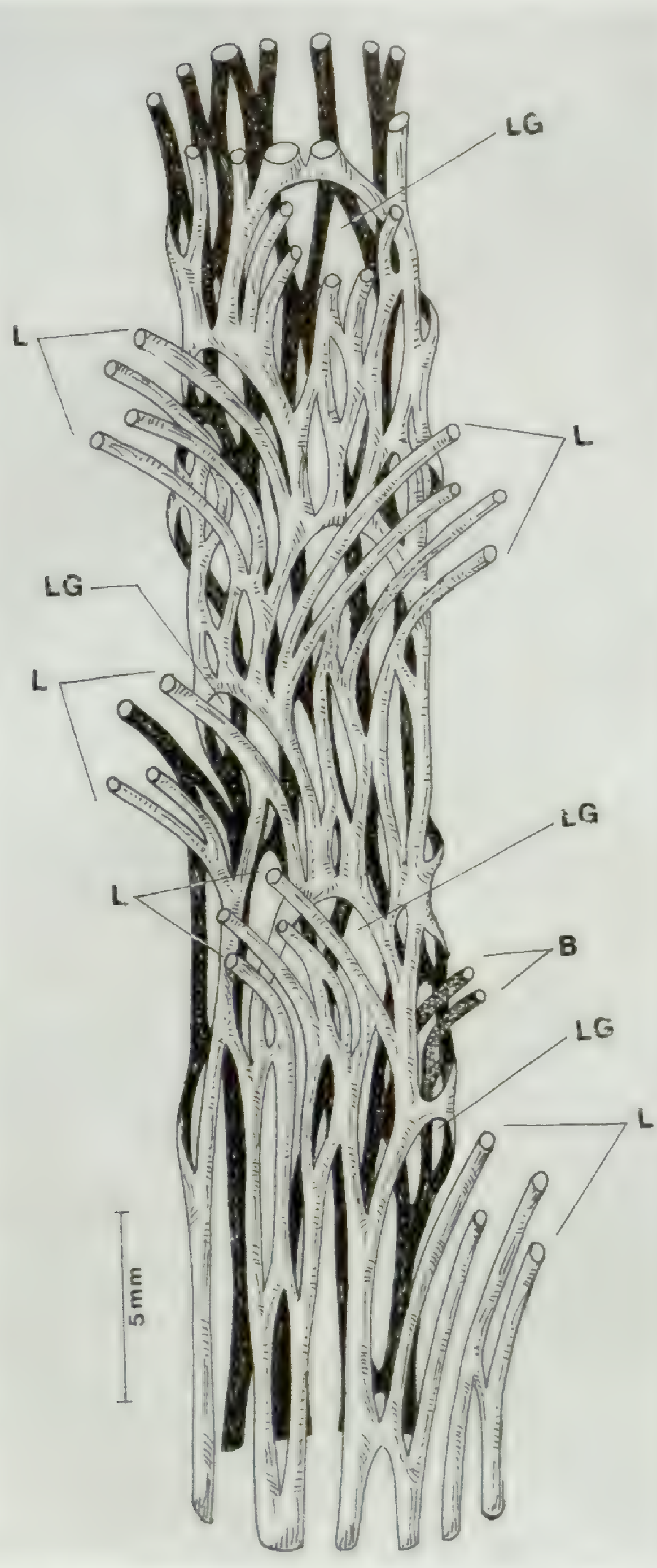
LEAF GAP.—Leaf gaps are usually oblanceolate in *P. fauriei* (Fig. 3, LG) and *P. interjectum* (Fig. 4, LG) with bluntly rounded anterior ends, while in *P. cambricum* (Fig. 1–2, LG) they are broad and ovate to oblanceolate with broadly rounded to bluntly rounded anterior ends. Leaf gaps of successive leaves overlap conspicuously and successive leaf gaps of the same side also overlap, though only slightly in *P. fauriei* and *P. interjectum*. In these two species the posterior end of each leaf gap extends downwards a little on either side of the anterior end of the next leaf gap so that the region between the two leaf gaps appears as a narrow, arched vascular strand.

In *Polypodium cambricum* and *P. sibricum* the leaf gaps extend only a short distance beyond the region where leaf trace separates (i.e., the leaf trace strands are given off from the margins close to the anterior end of the leaf gap), while in *P. fauriei* and *P. interjectum* the leaf gaps are longer, extending markedly beyond the region of separation of the leaf trace (i.e., the leaf trace strands are given off from towards posterior margin of the leaf gap) as also reported in *P. vulgare* (Srivastava and Khare, 2005). However, in *P. interjectum* some of the leaf gap extends only a short distance beyond the region where the leaf trace separates.

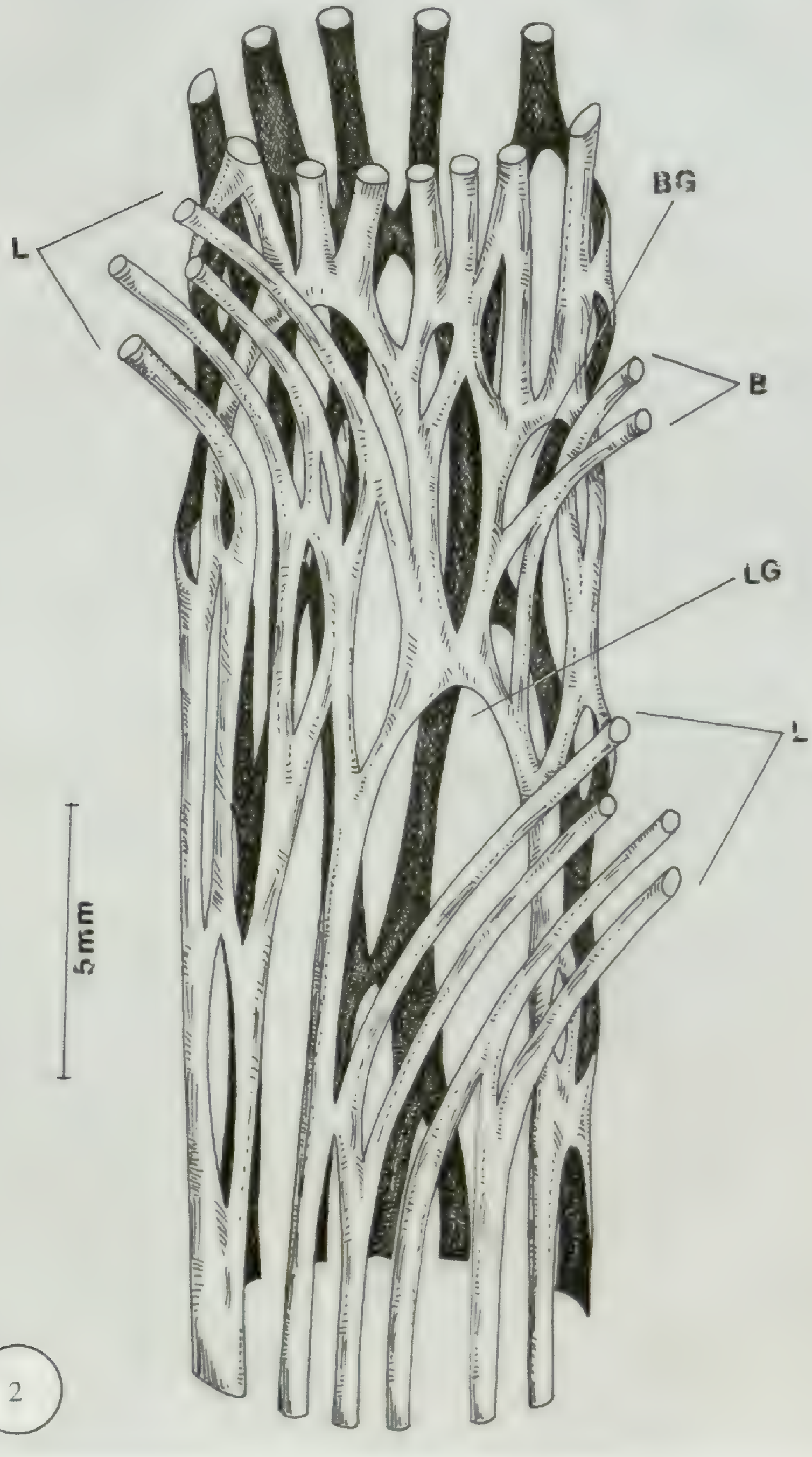
LEAF TRACE.—The leaf trace is highly dissected by profuse, short to elongated lacunae which are regularly placed towards the basal end of the leaf trace so that the basal region of the leaf trace often forms a closed-meshed reticulum. Each leaf trace is often composed of 4–8 slender vascular strands in the species of *Polypodium* studied. The leaf trace strands usually branch off from the margins close to the anterior ends of the leaf gaps in *P. cambricum* (Figs. 1–2, L) and *P. sibricum* while in *P. fauriei*, and *P. interjectum* leaf trace strands usually branch off from the posterior margins of the leaf gaps (Fig. 3–4, L) making the vascular strands of the leaf trace appear as independent strands traversing the cortex of the rhizome. As in other Polypodiaceae described, the adaxial margins of the leaf trace are slightly thickened (so that the last pair of leaf trace strands is thicker than the others).

BRANCH GAP.—Each branch trace has a conspicuous branch gap, placed next to the ventral posterior end of the leaf gap of the associated leaf (*P. cambricum* and *P. fauriei*) and conspicuously overlapping with the associated leaf gap though distinctly separated from it by a slender vascular strand (Figs. 1–3, BG). In *P. interjectum* (Fig. 4, BG) and *P. sibricum* the branch gaps are short, and intimately associated with the base of the leaf trace so that it appears to be a part of the reticulated base of the leaf trace (i.e., the branch gap is merged with the leaf gap becoming inconspicuous). However, in *P. sibricum* the branch trace is often solitary, becoming inconspicuous and appears to originate from the leaf trace strands themselves. Successive branch gaps on the same side do not overlap.

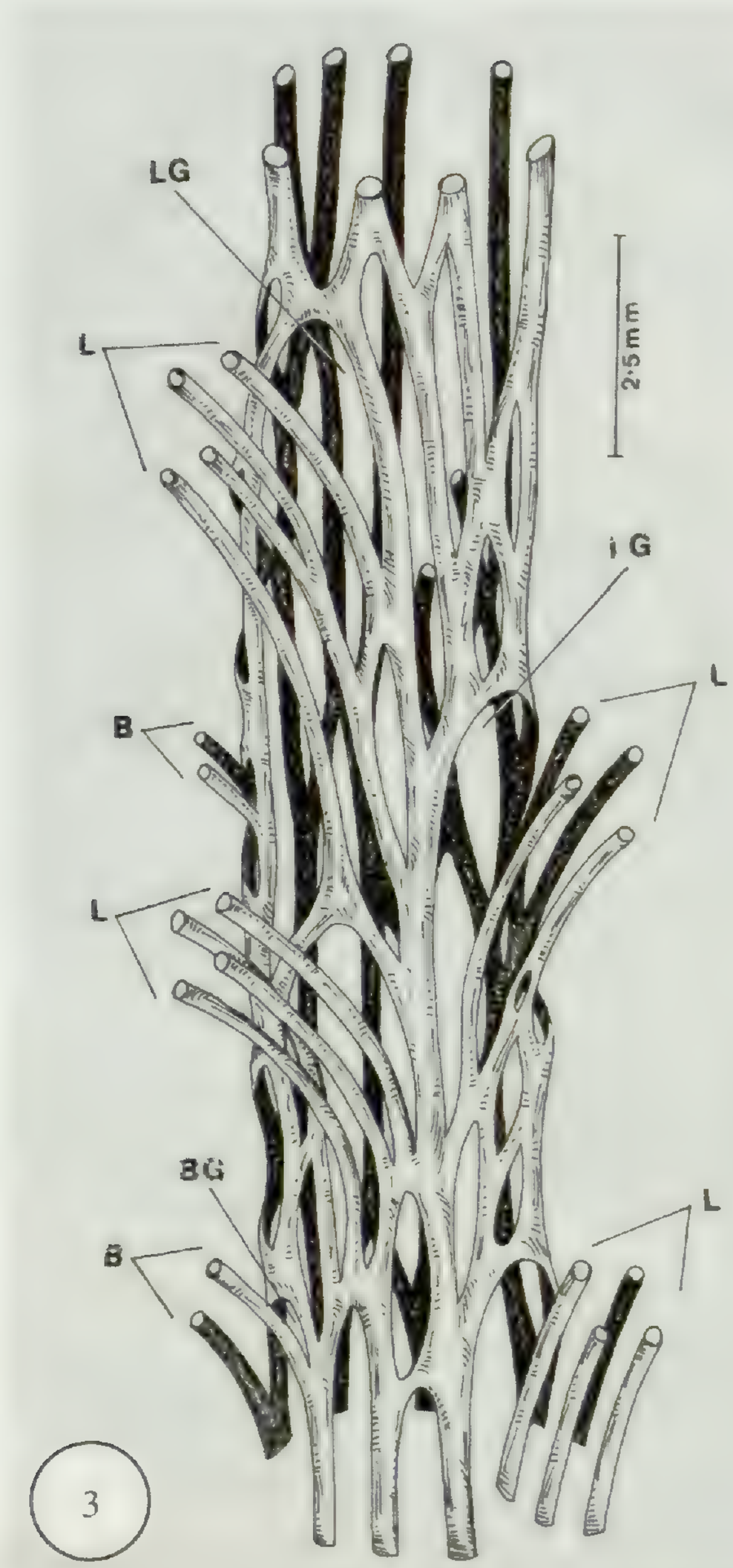
BRANCH TRACE.—As is characteristic of all Polypodiaceae, a branch is associated with a leaf at its abaxial side. Each branch trace is a simple structure, often composed of 2–4 slender, cylindrical vascular strands which do not form a

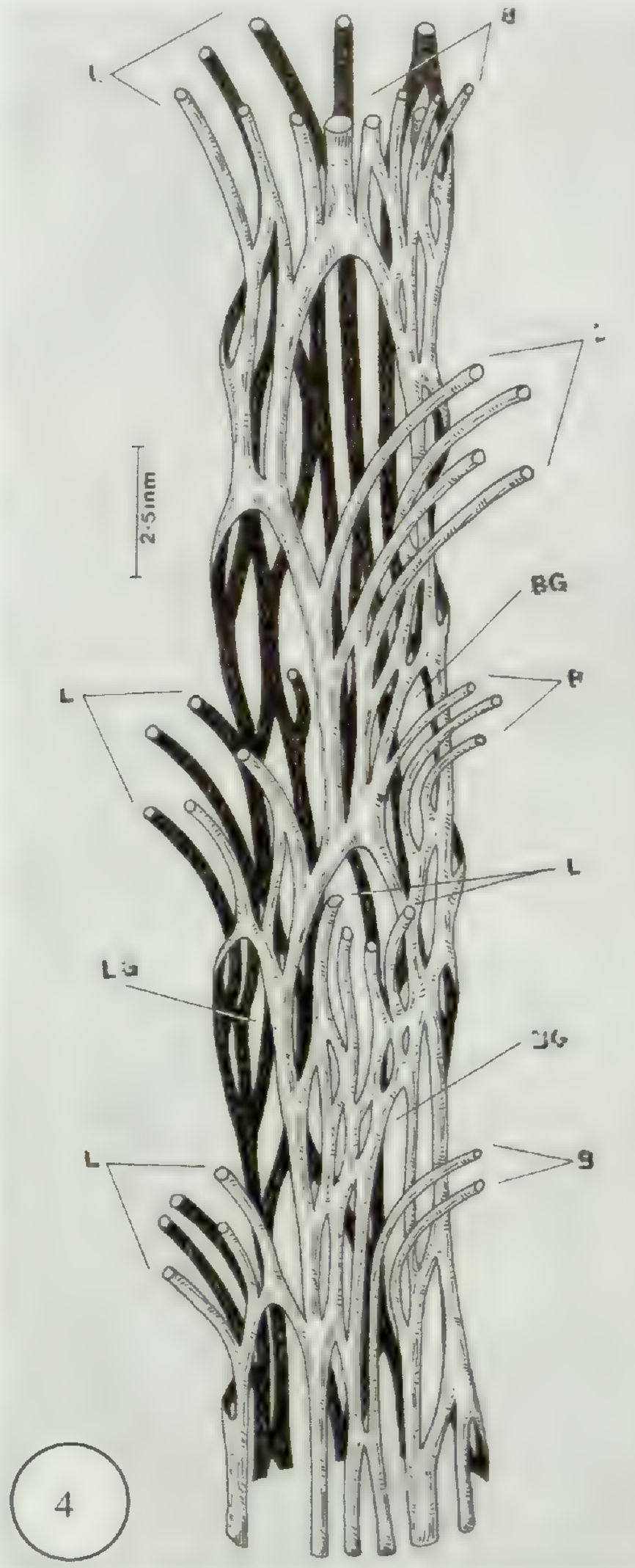


FIGS. 1–2. Vascular cylinder of a portion of the adult rhizome as seen from the dorsal surface. **1.** *Polypodium cambricum*; **2.** *P. cambricum* (*B*, branch trace; *BG*, branch gap; *L*, leaf trace; *LG*, leaf gap).



FIGS. 1–2. Continued.





FIGS. 3–4. Vascular cylinder of a portion of the adult rhizome as seen from the dorsal surface, **3.** *Polypodium fauriei*; **4.** *P. interjectum* (*B*, branch trace; *BG*, branch gap; *L*, leaf trace; *LG*, leaf gap).

reticulate vascular cylinder (Figs. 1–4, B). There is further reduction of the branch trace strands in *P. sibricum* where the branch trace is often solitary. Also, in some of the leaves there is no branch associated with the leaves (Figs. 1, 3, and 4).

Structure of the Vascular Cylinder

The vascular cylinder of the rhizome is composed of usually 9–12 slender and mostly cylindrical vascular strands. Xylem tissue of each vascular strand is ribbon-like and tracheidal, 1–5 cells thick in *P. cambricum* (Fig. 5), 2–5 cells thick in *P. fauriei* (Fig. 6), 1–5 cells thick in *P. interjectum* (Fig. 7), 1–3 cells

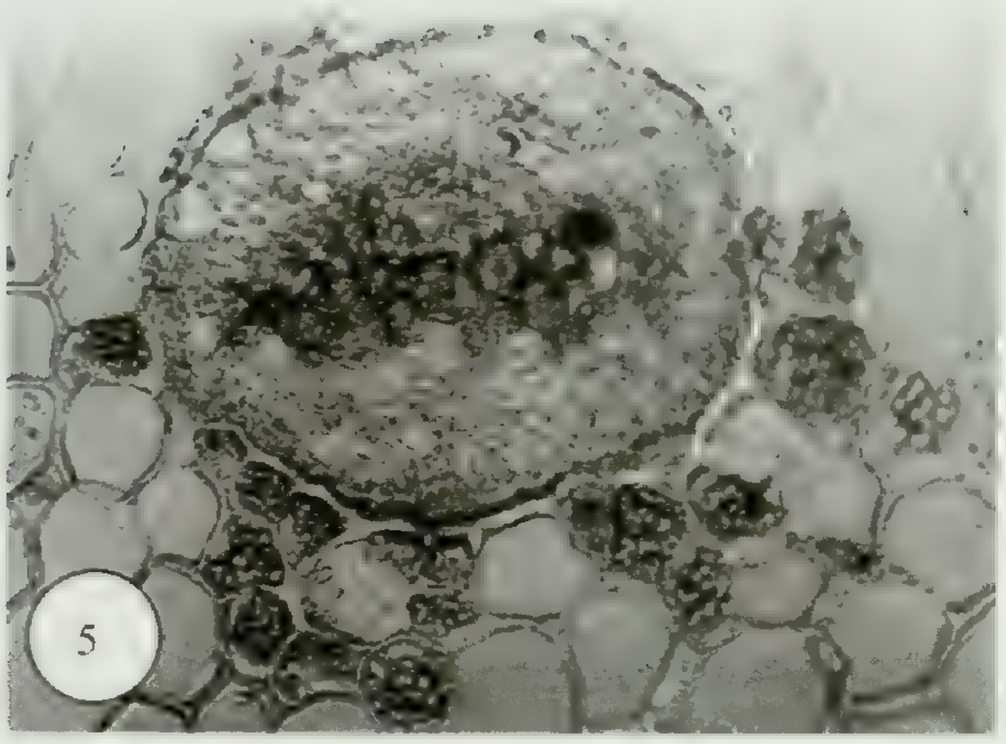
thick in *P. sibricum* (Fig. 8). The tracheids are interspersed with thin-walled individual cells or bands of xylem parenchyma in between in *P. cambricum*, *P. fauriei* and *P. interjectum* (Figs. 5–7) while in *P. sibricum* (Fig. 8), as in *P. vulgare* (Srivastava and Khare, 2005), the tracheids are not intermixed with xylem parenchyma. A thin sheath of 1–2 irregular layers of small parenchyma cells envelops the xylem tissue except at the free ends. The protoxylem is generally exarch in position, being present at the ends of the xylem tissue. The phloem is not continuous and is interrupted at the ends of the xylem tissue. Phloem is restricted to either surface of the xylem tissue and is interrupted at either end. It is massive, composed of 1–8 layers (*P. cambricum* and *P. fauriei*, 2–8 layers; *P. interjectum*, 2–4 layers; *P. sibricum*, 1–3 layers) of narrow, small, thin-walled parenchyma cells intermingled with few sieve cells. The pericycle is prominent, continuous around the vascular tissue, and consists usually of 1–2 layers of thin-walled, small regularly arranged polygonal parenchymatous cells. However, in *P. cambricum* and *P. fauriei* the pericycle is single layered consisting of broad, polygonal cells. The endodermis is well developed and composed of a single layer of rectangular, small, thin-walled cells with casparian thickenings on their radial walls. However, in *P. cambricum* the inner walls of the cortical parenchymatous cells abutting on the endodermis are usually thickened. Some of the endomermal cells have dark brown phlobaphene contents in their cells but there is no continuation in all the cells. The endodermis of some of the vascular strands are devoid of phlobaphene contents.

Discussion

Polypodium is a fern genus of the north temperate regions of the world with a few species in Africa and eastern Asia. Holttum (1947) regards the Polypodieae as derived by reduction from *Phymatodes*, while *Polypodium* is regarded as an ancestral genus evolved independently of Microsorieae and Pleopeltideae by Copeland (1947). Copeland (1947) kept *Polypodium* as separate genus, most numerous in American tropics.

In general, open venation (simple, free) represents an ancestral condition but in ferns, especially Polypodiaceae, the most ancestral members have a complex netted venation, the few species with free veins being derived. Thus, according to Holttum (1947, 1964) reversion has taken place in Polypodiaceae. Mickel (1982) also considers the veins in the more recently derived groups free.

The restricted distribution of free venation, found only in the *Polypodium*-group, suggests strongly that reticulate venation is an ancestral character in the Polypodiaceae. The north-temperate *Polypodium* does not represent the ancestral condition, but the late offshoot of a tropical stock. *Polypodium* species have free veins but are connected by intermediates with the species which have anastomizing veins (more ancestral members in *Polypodium* have anatomizing veins).



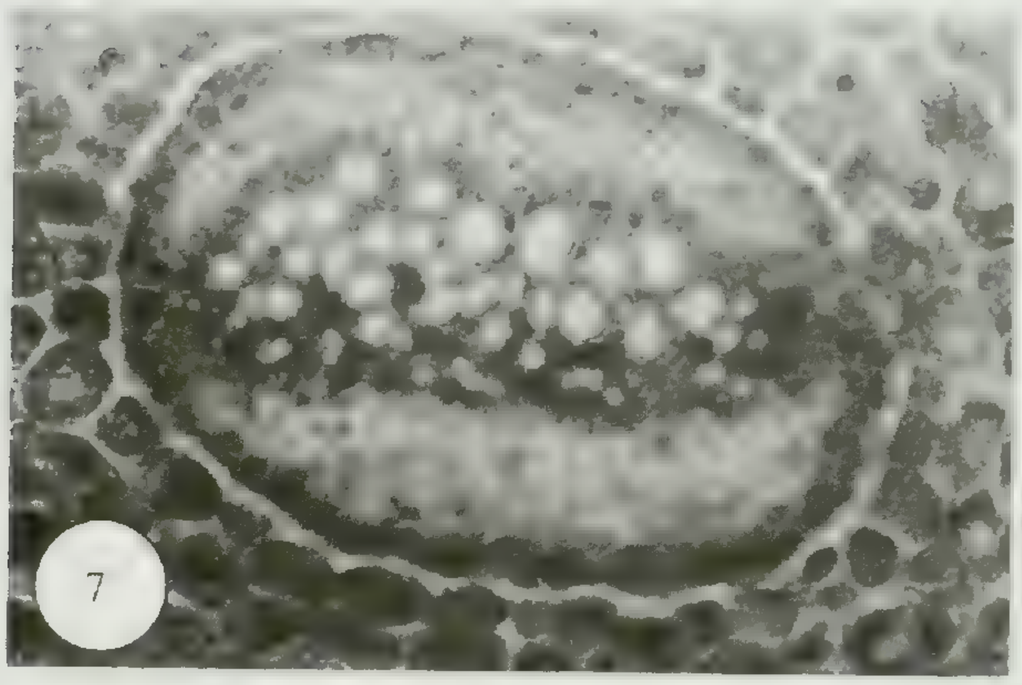
FIGS. 5–8. Transections of the adult rhizome showing cellular organization of a vascular strand. **5.** *Polypodium cambricum*; **6.** *P. fauriei*; **7.** *P. interjectum*; **8.** *P. sibricum* (× 400).

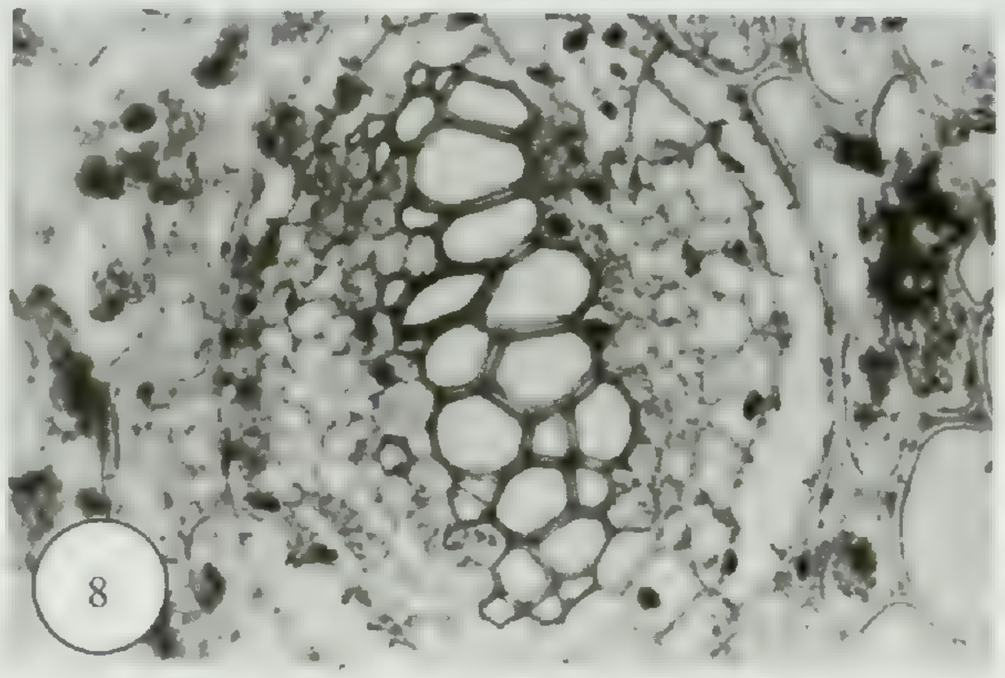
In contrast to *P. vulgare* (Srivastava and Khare, 2005) the dorsal median vascular strand is distinct and slightly thicker than other vascular strands of the rhizome in all the four species studied here. Recently, Smith *et al.* (2006) stated that the xylem is usually mesarch in the shoot of Monilophytes (including Eu- and Leptosporangiate ferns). However, contrary to the above observations, the protoxylem is restricted to either narrowed margins (lobes) of the xylem band on the side facing the cortex of the rhizome i.e., protoxylem is exarch in all of the species studied.

Each leaf trace in the *Polypodium* species is highly dissected and usually composed of 4–5 slender vascular strands while in *P. sibricum* it consists of 5–8 vascular strands. In contrast to *P. vulgare* (Srivastava and Khare, 2005) the leaf trace strands usually branch off from the margins close to the anterior end of the leaf gaps in *P. cambricum* and *P. sibricum* while in *P. fauriei* and *P. interjectum* the leaf trace strands branch off from the posterior margin of the leaf gaps. However, in *P. interjectum* some of the leaf gap extends only a short way beyond the region where the leaf trace separates. In *P. cambricum* and *P. sibricum*, the leaf gaps extend markedly on the posterior end of the leaf trace as found in the comparatively primitive species. In other species such as *P. vulgare* (Srivastava and Khare, 2005) and *P. interjectum* the leaf gap extends only slightly on the posterior side of the leaf trace.

Branch gaps are comparatively very short and often merged with the leaf gaps becoming inconspicuous (*P. interjectum*). There is further progressive reduction of the branch trace strands in *P. sibricum*. The branch trace is often solitary without any branch gap and is often merged, becomes inconspicuous, and appears to originate from the leaf trace strands themselves; thus forming an integral part of the leaf trace.

Perhaps the most interesting morphological feature of the rhizome is seen in *P. interjectum* and *P. sibricum* where the characteristic association of branch trace/gap with the leaf trace/gap is observed. In some cases the branch trace/gap is often merged with the leaf trace/gap, becoming inconspicuous and forming an integral part of the leaf trace/gap. Such type of association has also





FIGS. 5–8. Continued.

been reported in *P. vulgare* (Srivastava and Khare, 2005) and *Pleopeltis tweediana* (Hook.) A. R. Smith (Srivastava and Chandra, unpublished data). The absence of associated branches with some leaves and the closer associations of leaves and branches in *P. interjectum* and *P. sibricum* seem to be significant and may indicate the evolutionary status of the taxa. Possibly the condition where the branch gap/trace extends to the leaf gap/trace is relatively more advanced than where the branch gap/trace is distinct and lateral to the leaf gap. Based on this it has been suggested that the vascular architecture of the rhizome in *Polypodium* species studied possibly exhibits a derived condition. However, the evolutionary significance of this association between leaf and branch needs more extensive investigation.

Relationship to Goniophlebium.—*Goniophlebium* was regarded by some taxonomists as congeneric with *Polypodium* (Christensen,1938; Holttum,1949; Hennipman *et al.*,1990). However, many prefer to treat it as separate genus (Ching, 1940; Copeland, 1947; Holttum, 1968; Pichi-Sermolli, 1977; and Srivastava and Khare, 2005) most numerous in American tropics and maintain it as comparatively ancestral and intimately related to *Polypodium*. However, Tryon and Tryon (1982), considered *Goniophlebium*, an Old World genus with articulate pinnae, as not closely related to *Polypodium s.s.*, which is predominantly a New World genus.

Based on molecular studies, Schneider *et al.* (2004) showed that *Goniophlebium* is more distantly related to *Polypodium* than has been suggested. They further indicated that *Goniophlebium* is part of a large Old World clade that includes various genera such as *Lecanopteris*, *Lepisorus*, and *Microsorum*. They also provided evidence for a monophyletic *Goniophlebium*, as defined by Rodl-Linder (1990).

The differences in the vasculature of the rhizome observed here do not support a close association of *Polypodium* with paleotropic *Goniophlebium*. The results are consistent with the studies from molecular analyses (Schneider *et al.*, 2004), which suggested that *Goniophlebium* is more distantly related to *Polypodium* than previously suggested. In contrast to *Goniophlebium* (Srivastava and Khare, 2005) *Polypodium* species possess 1) dorsal median vascular strands scarcely different from other vascular strands, 2) usually

obliquely placed leaf gaps, 3) Leaf trace usually with four vascular strands, 4) much reduced branch trace usually with 1–3 vascular strands, 5) narrow branch gap which is less than half as long as the leaf gap, 6) Branch trace/gap in some cases merge with the leaf trace/gap forming integral part of it, 7) in some cases no branch associated with leaves, and 8) no sclerenchyma strands in the ground tissue. Until details regarding a large number of *Polypodium* species become available, an evaluation of the significance of stelar architecture in this group will not be possible.

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Isoetes maxima, a New Species from Brazil

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Abstract.—*Isoetes maxima* from eastern Brazil is described as a new species. This taxon differs from other fully aquatic species in South America by a combination of its overall size, leaf coloration, finely tapering subulae, and megaspore morphology.

Key Words.—*Isoetes maxima*, Brazil, new species

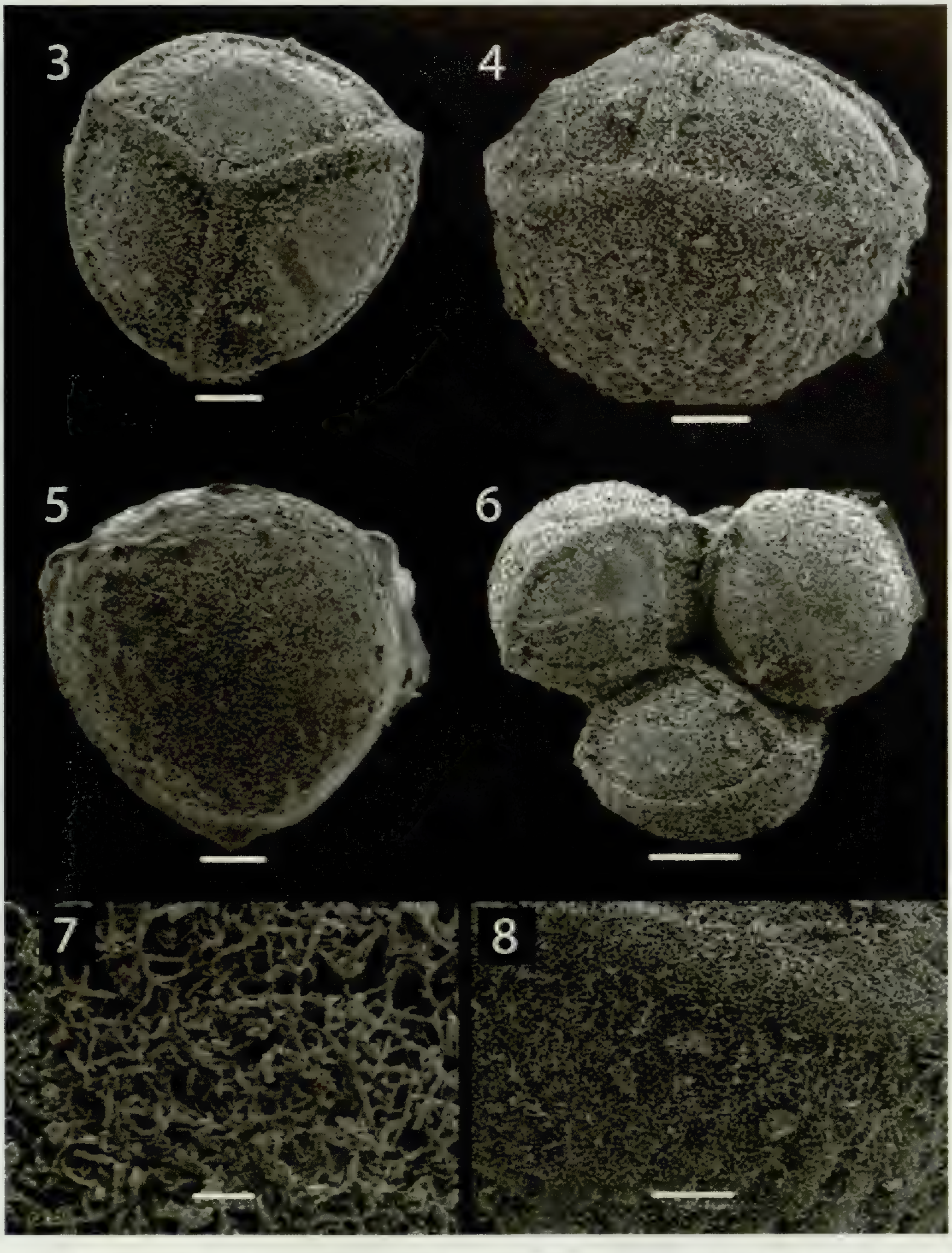
While examining *Isoetes* from eastern Brazil, we encountered a specimen whose large size and dark coloration are unlike any known species from that region. The plants have numerous, very narrow, densely packed, and finely tapering leaves, and its megaspores are rugulate to tuberculate. In contrast, other large *Isoetes* in this region of South America have broader leaves that are less densely packed, and reticulate megaspores (Fuchs-Eckert, 1986; Macluf *et al.*, 2008).

The specimens in question were collected by Aloysio Sehnem in 1970. Sehnem determined his collection to be a new species and designated it so on the label as *Isoetes maxima*. The label indicates the collection as the intended holotype; however, Sehnem never described or validly published the new species. We have elected to use the name that Sehnem had inscribed on the specimen label since the overall stature and robust appearance of the taxon merit the appellation.

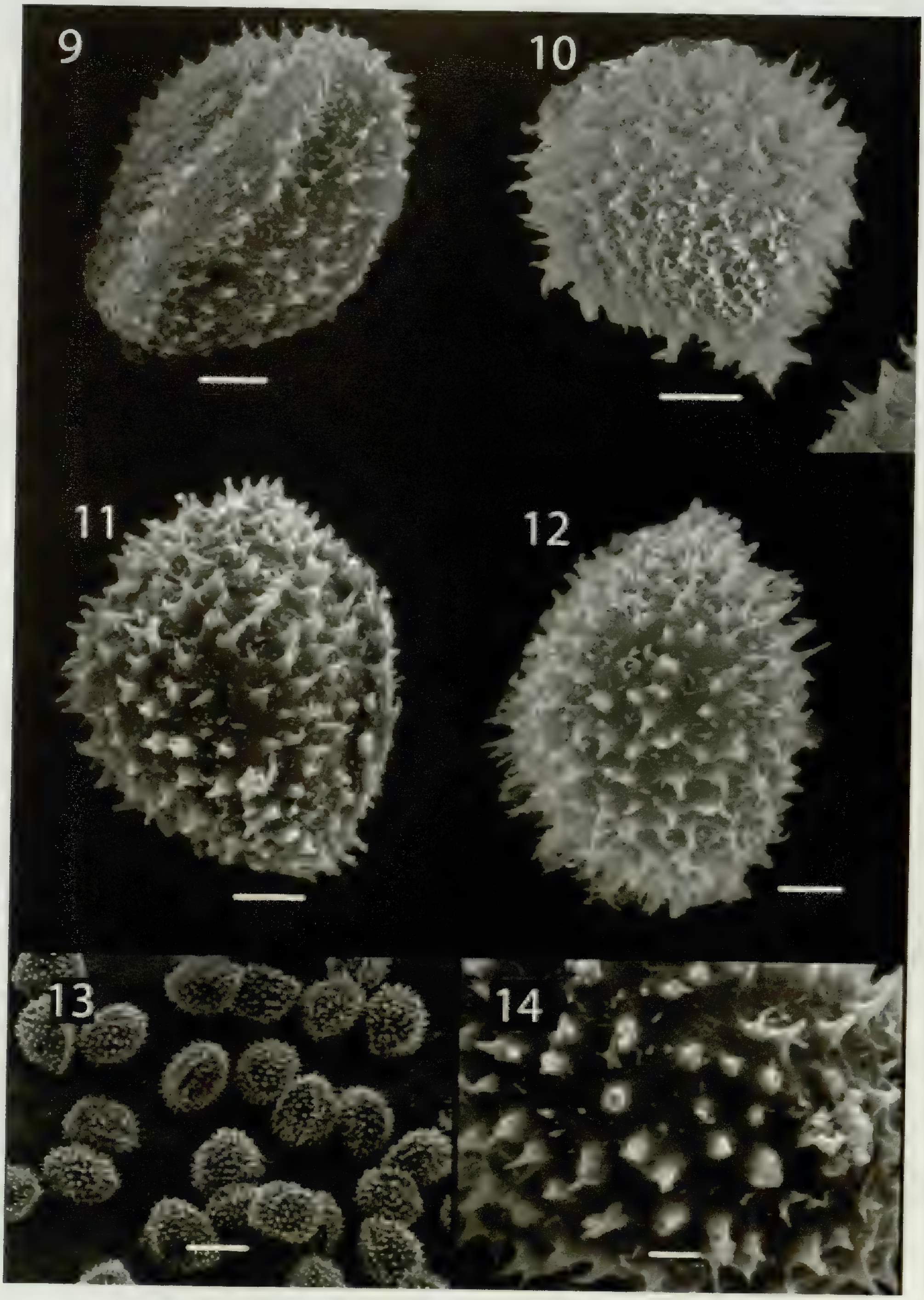
Isoetes maxima Hickey, Macluf and Link-Pérez, ***sp. nov.*** TYPE.—BRAZIL. Cambará, Fortaleza, Aparados, in aqua rivi in campo, 1200 m, 2/5/70, *A. Sehnem 10960* (PACA 74904). (holotype: PACA; isotypes: FHS). **Figs, 1–14.**

Cormus erectus, bilobatus, 2.5–3.0 cm latus; *radices* succulentae, dichotomae. *Foliae* 30–50, ad 45 cm longae, strictae, erectae, 1.5–2.0 mm latae ad medium, ca 8–10 mm latae basi; *alae* atrovirentes vel fuliginosae, 2.0 mm latae ad sporangium, 10–25 cm longae (20–50% per foliae longitudinem ascendentes), apice attenuato; *subula* atrovirens, erecta, semi-teres, apice longiattenuato, neque nitido neque corneo; *fasiculi* fibrosi peripherici praesentes; *squamellulis* carentibus. *Labium* triangule, 850–875 µm altum, 475–500 µm latum. Ligula magna, auriculata manifeste; pulvinus residuus triangularis, persistens, ca 2 cm longum et 1.5 mm latum. *Velum* incompletum, 30–50%

FIGS. 1–2. Holotype of *Isoetes maxima* (*Sehnem 10960*, PACA). 1. Whole plant. 2. Adaxial views of two sporophylls with microsporangia, partial velum and ligule fragments.



FIGS. 3–8. Scanning electron micrographs of *Isoetes maxima* megaspores (*Sehnem 10960*, PACA). 3. Proximal view of a megaspore. 4. Equatorial view of a megaspore. 5. Distal view of a megaspore. 6. Three of the four spores of a single tetrad. 7. High magnification of the distal surface. 8. Detail of the equatorial zone, with both the equatorial ridge and a weak girdle visible. Fused tubercles, rugae, are visible in Figs. 4, 5 and 8. Scale bar = 100 μm in Figs. 3–5; 200 μm in Fig. 6; μm in Figs. 7 and 8.



FIGS. 9–14. Scanning electron micrographs of *Isoetes maxima* microspores (*Sehnem 10960*, PACA). 9. Proximal view. 10. Equatorial view showing the supra-laesural expansion. 11. Equatorial view. 12. Distal view. 13. Several microspores in different views. 14. High magnification of the distal echinate ornamentation. Scale bar = 5 μm in Figs. 9–12, 20 μm in Fig. 13, and 2.5 μm in Fig. 14.

per sporangium longitudinem descendens. *Sporangium* basale, ellipticum, hyalinum, 5–6 mm longum, 2–4 mm latum, non maculatum. *Megasporae* cretaceae, triletae, 525–(583.8)–650 μm diametro, globosae, *pagina* distalis verrucata vel tuberculata (vel laevis), *pagina* proximalis laevis, *cingulum* leave. *Microsporae* brunneae vel atrobrunneae, monoletae, 27–33 μm longae, 20–23 μm latae, echinatae.

Plants large, corms erect, bilobed, 2.5–3.0 cm across. Roots numerous, succulent, dichotomously branched. Leaves 30–50, to 45 cm long, straight, erect, 1.5–2.0 mm wide at mid-length, ca 8–10 mm wide at the base; alae dark green to brown, 2.0 mm wide at the sporangium, extending 10–25 cm up the leaf (20–50% of total leaf length), apex attenuate; subula dark green, erect, appearing half-terete, apex long attenuate, neither glossy nor corneous, fibrous bundles present; scales absent. Labium triangular, 850–875 μm high, 475–500 μm wide. Ligule large, massive, distinctly auriculate; cushion dark, persistent, triangular, ca 2 mm long, 1.5 mm wide. Velum extending down and covering 30–50% of the sporangium; lower velum also present and covering 5–10% of sporangium. Sporangium basal, elliptic, hyaline, 5–6 mm long, 2–4 mm wide, concolorous. Megaspores dull white, trilete, 525–(583.8)–650 μm in equatorial diameter, distal surface rugulate to tuberculate (to laevigate), comprised of an open reticulum of fibrils, proximal surfaces laevigate, subtriangular to globose in polar view and globose in equatorial view, laesurae 44.5 μm high, equatorial ridge 30 μm wide, girdle typically smoother than the distal ornamentation. Microspores brown to dark brown *en masse*, monolete, 27–33 μm long, 20–23 μm wide, elliptic in polar view, proximal face convex and distal face broadly rounded, perispore surface echinate with longer echinae distally.

PARATYPES.—BRAZIL. **Itaimbezinho**, Sao Francisco do Paulo, in stagno ad flumen Perdizes, alt. 900 m, Dec. 24, 1980, *A. Sehnem 17148* (PACA 74905). **Cambará**, Fortaleza, in rivulo submersum in lectu, alt. 1000m, Jan 10, 1973. *A. Sehnem 12362* (PACA 74906).

Isoetes maxima is an aquatic endemic known only from three collections out of the Cambará region of Rio Grande do Sul in Brazil. It grows submerged in streams at elevations of 900 to 1200 m.

Species of *Isoetes* from Rio Grande do Sul have either reticulate or tuberculate-rugulate to rugulate megaspores. The reticulate-spored species of South America are in desperate need of revision. Those species of eastern and southern South America (from Minas Gerais to Buenos Aires) constitute an exceptionally difficult assemblage, having had less attention paid to them than the reticulate-spored species of the northern Andes. Fuchs-Eckert (1986) treated the reticulate-spored species of this region, focusing primarily on the State of Santa Catarina. Although most non-Andean reticulate species were covered, these taxa are still imperfectly differentiated.

The only two non-reticulate species in southeastern Brazil are *Isoetes weberi* Weber and *Isoetes maxima*. Megaspore ornamentation in both species varies from tuberculate to rugulate, but the species differ dramatically in megaspore

size: *Isoetes weberi* spores have a mean of 356 µm and a range of 220 to 450 µm; those of *I. maxima* range from 525 to 650 µm with a mean of 583 µm. At high magnifications, the megaspore surfaces of both species consist of a coarse open reticulum of structural elements. This type of surface is not seen in any of the reticulate spored species. Microspores in both species are fundamentally echinate, but in *I. weberi* the echinae are broader, more columnar and distally muricate (Hickey, 1985). Both species have moderately well developed vela extending about 50% down the sporangium. The species also differ markedly in the size and shape of the labium. In *I. maxima* the labium is narrowly triangular, with a length to width ratio of about 1.8 to 1 and reaching lengths of 0.85–0.88 mm in height. The labium of *I. weberi* ranges from 1.0–1.8 mm in height, is narrowly oblong and has a length to width ratio of 2.3–3.3. The labium of *I. weberi* is typically bifid distally, a condition otherwise known only in *I. tennesseensis* Luebke and Budke (Budke *et al.*, 2005).

Isoetes weberi is a lowland species growing at elevations of 10–20 m whereas *I. maxima* is found at about 1200 m. The presence of scale leaves around the corms in *I. weberi* suggests that it frequents drier habitats with only seasonal inundation, and so differs from the more aquatic *Isoetes maxima*.

The rather large megaspores seen in *Isoetes maxima* suggest an origin through polyploidy. Within the vicinity of *I. maxima* only *I. weberi* stands as a likely parent. In fact, the similar morphology and habit in conjunction with the common, open reticulum of the megaspores suggest an affinity between these two. Candidates for a second parent are more problematic and perhaps must be sought further north in the states of Bahia, Espirito Santo and Rio de Janeiro, perhaps to the poorly understood *I. organensis* Weber or *I. ulei* Weber.

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New Records of *Polyphlebium borbonicum*, an African Filmy Fern, in the New World and Polynesia

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ABSTRACT.—*Polyphlebium borbonicum* is newly recorded in Central and South America and Easternmost Polynesia (Marquesas and Society Islands). It has been misidentified as *P. diaphanum* and as *P. endlicherianum* in the New World and in the Pacific, respectively. *Polyphlebium borbonicum* is distinguishable from true *P. diaphanum* by broader blade segments, and from true *P. endlicherianum* by the absence of a marginal elongate cell row of the lamina.

KEY WORDS.—distribution, Hymenophyllaceae, Marquesas

In the course of preparing a treatment of Hymenophyllaceae for the *Vascular Flora of the Marquesas Islands* project, it came to our attention that specimens identified as *Trichomanes endlicherianum* C. Presl (= *Polyphlebium endlicherianum* (C. Presl) Ebihara & K. Iwats.) comprise two quite different forms. The first form has lanceolate fronds with an obvious row of clear elongate cells along the margins (Figs. 1a, 2d), and resembles typical *P. endlicherianum* distributed in the South Pacific. The second form has larger and broader fronds, an ovate outline, and no clear marginal cell row (Figs. 1b, 2a). So far, no species matching the characters of the second form has been recorded in the South Pacific area. From a global viewpoint, this form best matches *Polyphlebium borbonicum* (Bosch) Ebihara & Dubuisson, an African species. *Polyphlebium borbonicum* was originally described based on a specimen from Bourbon Island (La Réunion) and is widely distributed in tropical Africa (Beentje, 2008; Kornaś, 1994), but has not been recorded in either the New World or in Polynesia (with the exception of a single occurrence of *P. borbonicum* recently noted by Nitta (2008) in Moorea, French Polynesia; this specimen has been included in the current analysis).

We also noticed that some New World plants usually identified as *Trichomanes diaphanum* Kunth resemble both *P. borbonicum* and the

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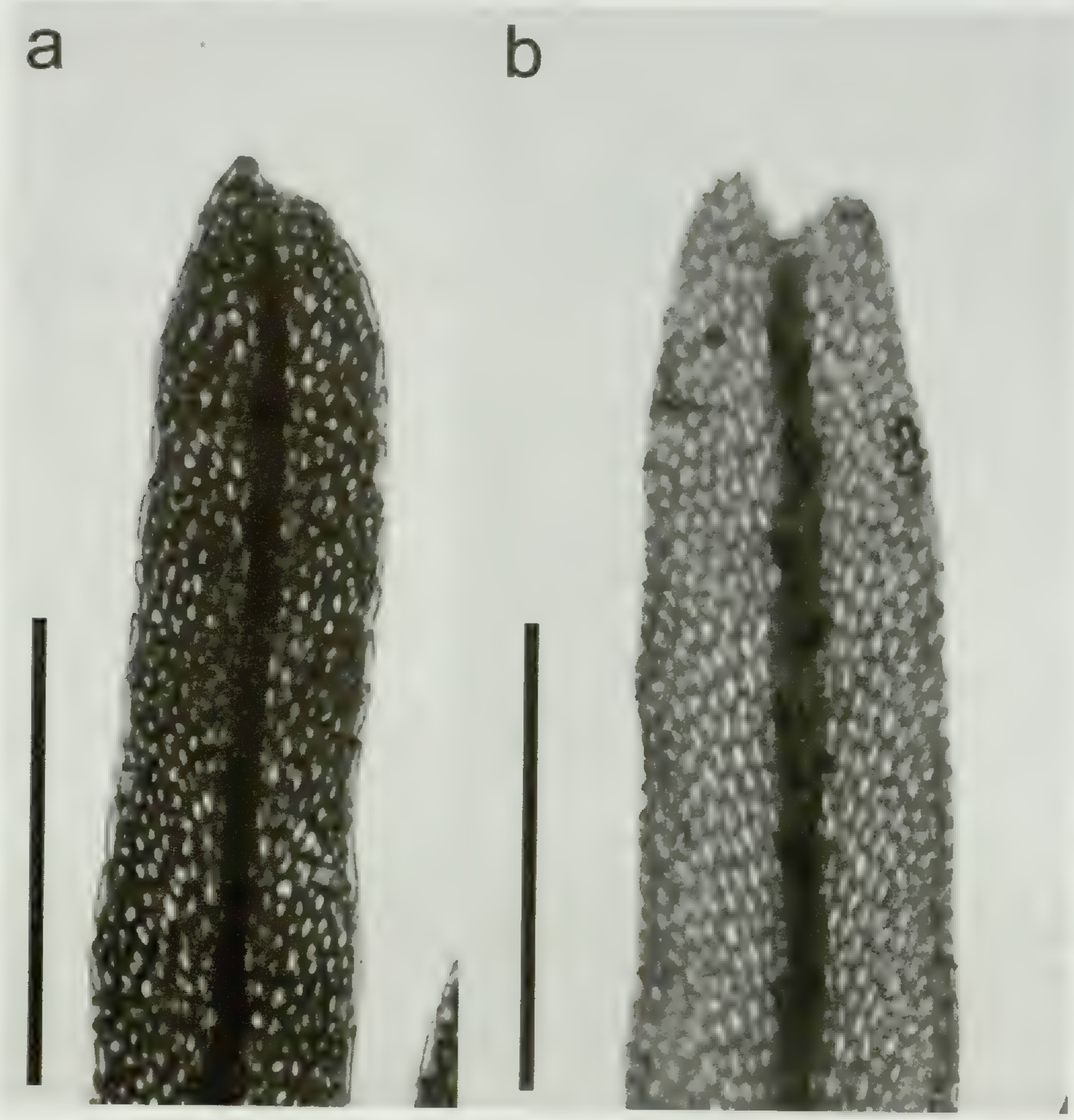

FIG. 1. Laminar cells of two Marquesas species. a. *Polyphlebium endlicherianum* (D. Lorence 9127, PTBG) with transparent marginal elongate cells; b. *P. borbonicum* (K. R. Wood 10500, PTBG) without marginal elongate cells. Scale: 1 mm.

unidentified Polynesian plant. Examination of 1077 base pairs (bp) of chloroplast *rbcL* sequences (methods of DNA and phylogenetic analyses followed Ebihara *et al.*, 2005), suggests that *P. borbonicum* of Réunion is the species most closely related to Polynesian *P. endlicherianum*. All samples examined in this study (Table 1) form a robustly supported monophyletic clade together with *P. borbonicum* of Réunion (Fig. 3). Based on both morphological and genetic homologies, we treat these disjunct distributed plants as a single species, *Polyphlebium borbonicum*.

The genus *Polyphlebium* as redefined by Ebihara *et al.* (2006) is comprised of about 15 species, located mainly in the southern hemisphere, with no species widely distributed across multiple continents. Since *P. borbonicum* has been misidentified as *P. endlicherianum* in Polynesia, and was submerged under the wider morphological variation of the *P. diaphanum* – *P. hymenophylloides* complex in the New World (Table 2), with proper

FIG 2. a–c. Specimens of *Polyphlebium borbonicum*. a. Marquesas Islands (K. R. Wood 4522, PTBG 24332); b. Bolivia (M. Kessler et al. 11435, UC 1621639); c. Réunion (J.Y. Dubuisson HR1999-22, P); d. a specimen of *P. endlicherianum* from the Marquesas Islands (D. Lorence 9127, PTBG). Scale: 1 cm.

identification, we propose that *P. borbonicum* is widely distributed across the paleotropics and neotropics (Fig. 4).

Polyphlebium borbonicum of the New World is recognizable by its segments of unequal length and broader fronds (more than 3 cm wide) in most cases. *Trichomanes debile* Bosch, a name long overlooked and synonymized under *P. diaphanum* (e.g., Lellinger 1989), has recently been applied by A. R. Smith to specimens having flat wings and less developed pinnae (pinnules at the basiscopic side are usually unbranched) at an acute angle against the rachis (identification was made for the herbarium specimens of UC) (Fig. 1b). Our result (Fig. 3) showed that one of two samples of *T. debile* is nested in the clade of *P. borbonicum* and that the other is closely related to the clade. We here advocate a taxonomic treatment synonymizing *T. debile* under *P. borbonicum*.

TABLE 1. Samples of *Polyphlebium borbonicum* used for phylogenetic analysis. [1]Sequences newly obtained for this study.

Original identification	Locality	Accession	Voucher (Herbarium)
P. borbonicum	Réunion	AY175782	Dubuisson HR1999-2 (P)
"*P. endlicherianum*"	Society Islands, Moorea	EU122988	Nitta 073 (UC)
"*P. endlicherianum*"	Marquesas Islands, Ua Huka	AB445233[1]	Wood 10501 (PTBG)
"*T. debile*"	Bolivia, Prov. Sud Yungas	EU784118[1]	Kromer 1753 (UC)
"*T. debile*"	Bolivia, Prov. Ayopaya	EU784117[1]	Jimenez 1568 (UC)

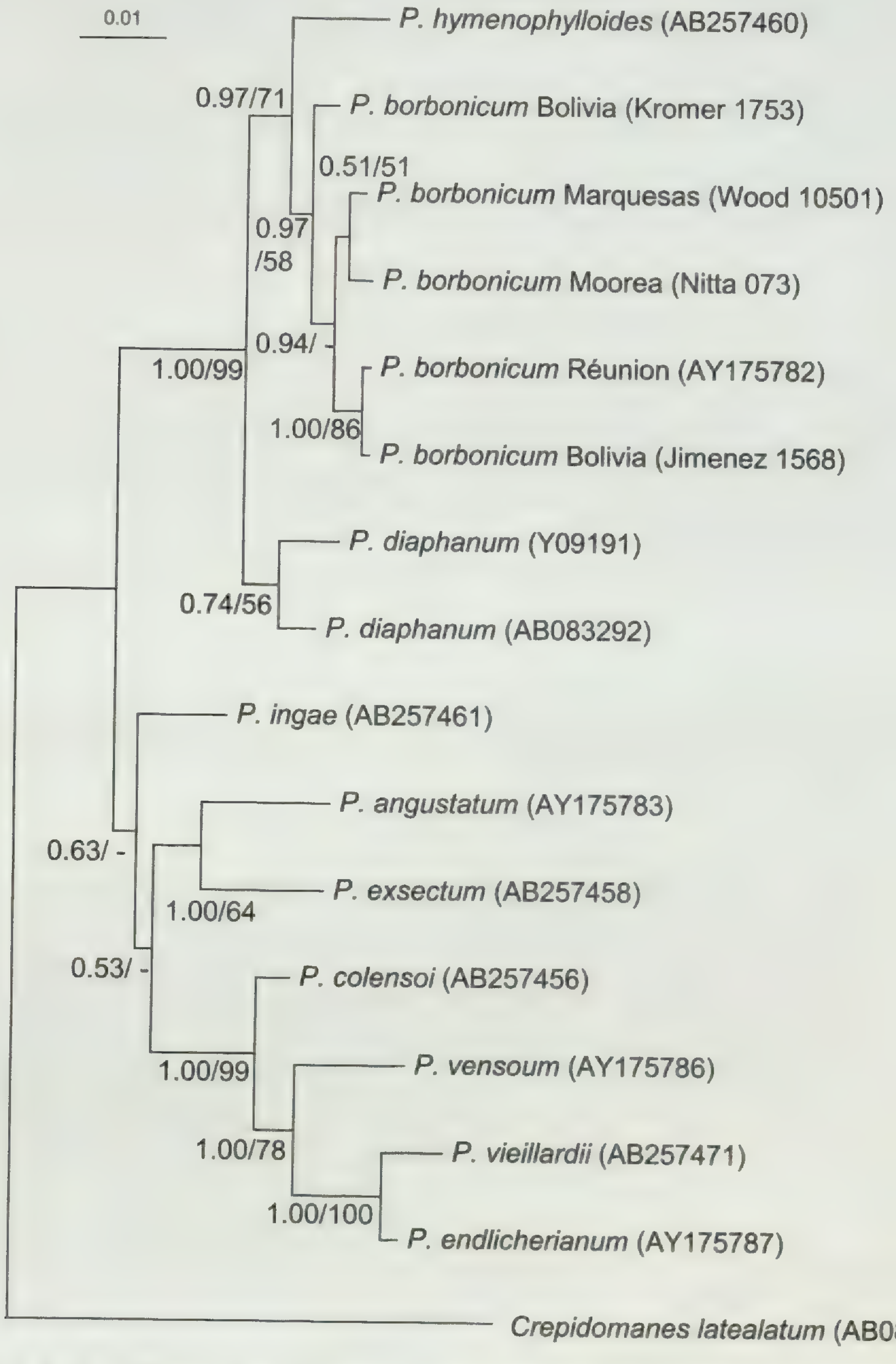

FIG. 3. A phylogenetic tree of Bayesian inference based on 1077bp of chloroplast *rbcL* sequences. Numbers at the nodes indicate support values (Bayesian posterior probability/maximum parsimony bootstrap).

TABLE 2. A comparison of morphological characters of *Polyphlebium borbonicum*, *P. diaphanum* and *P. endlicherianum*.

Character	*P. borbonicum*	*P. diaphanum*	*P. endlicherianum*
Marginal elongate cells	Absent	Absent	Present
Width of ultimate segments	0.8–1.0 mm	0.4–0.8(–1.0) mm	0.5–1.0 mm
Wings of rachis	Flat	More or less waved	Flat

TAXONOMIC TREATMENT

Polyphlebium borbonicum (Bosch) Ebihara & Dubuisson, Blumea 51: 240, 2006. *Trichomanes borbonicum* Bosch, Ned. Kruidk. Arch. 5(2): 158, 1861. *Vandenboschia borbonica* (Bosch) G.Kunkel, Nova Hedwigia 6: 213, 1963.

TYPE.—Ins. Borboniae, Boivin 908 (holotype: L? not seen; isotype: B). Fig. 2 a–c

Trichomanes debile Bosch, Ned. Kruidk. Arch. 5(2): 154, 1861.

TYPE.—VENEZUELA. Prov. de Carabado, 700 m, May 1846, Funck & Schlim 596 (holotype: L? not seen; isotype: UC).

Rhizomes long-creeping, frequently branching, filiform, less than 0.5 mm in diameter, densely covered with brown hairs, roots few and fine. Stipes (0.8–) 2–6 cm long, at a distance from the adjacent ones. Blades bipinnatifid-

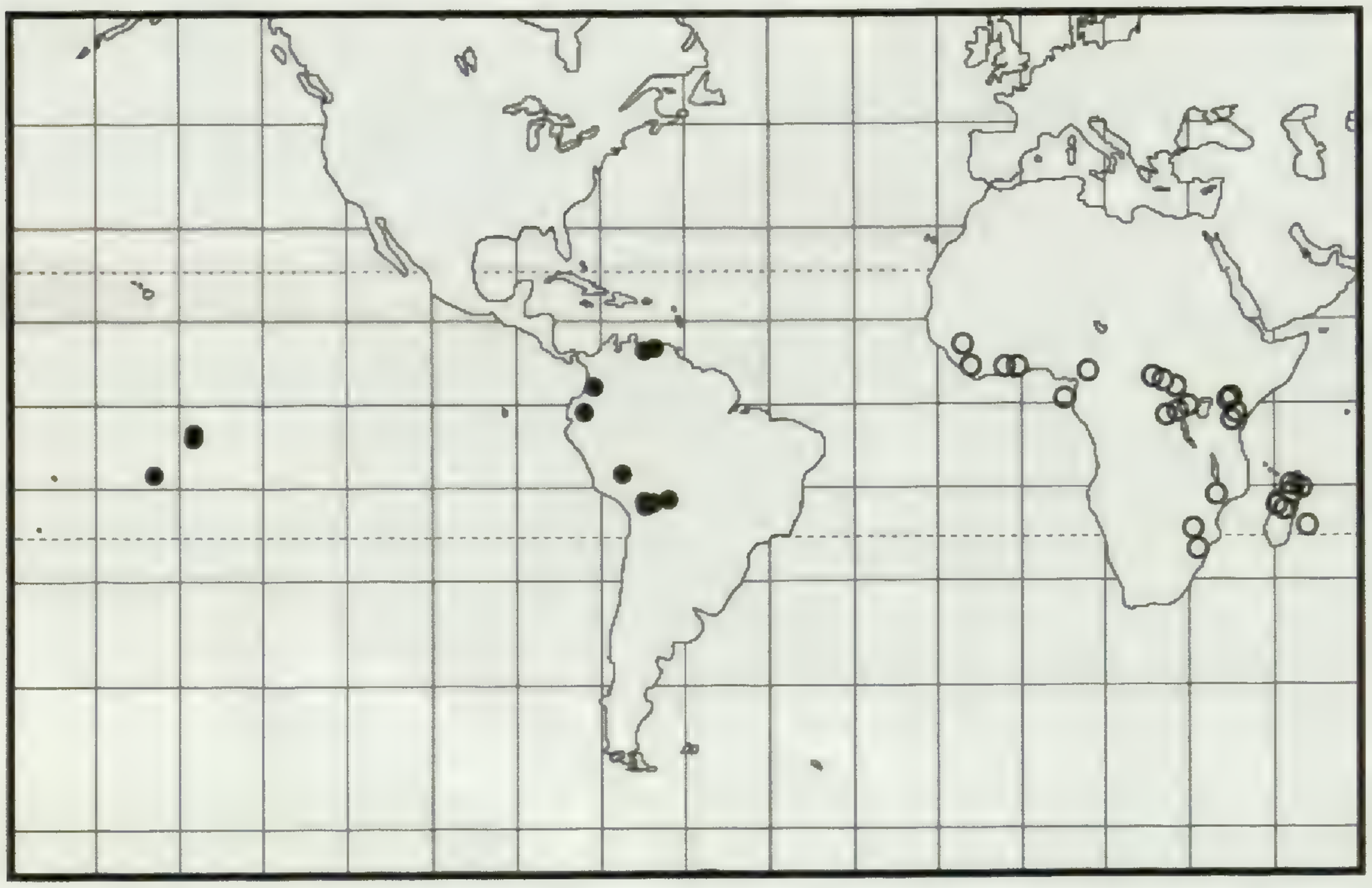

FIG. 4. A distribution map of *Polyphlebium borbonicum*. Black circles: new distributions by present study. White circles: previous records (Tardieu-Blot 1951, Kornaś 1994, Beentje, 2008).

bipinnate, ovate to lanceolate, to 18 cm long and 5.5 cm wide, ultimate segments 0.8–1.0 mm wide, venation anadromous, elongate marginal cells absent, false veinlets absent, internal cell walls thin and straight. Sori paratactic, tubular, lips dilate, receptacle exserted.

DISTRIBUTION.—Mascarene Islands, Madagascar, Continental Africa, Costa Rica, Colombia, Venezuela, Ecuador, Peru, Bolivia, Marquesas Islands, Society Islands (Moorea) (Fig. 4).

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Aspects of Gametophyte Development of *Dicksonia sellowiana* Hook (Dicksoniaceae): an Endangered Tree Fern Indigenous to South and Central America

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Abstract.—With the purpose of providing a basis for programs of sustainable management in the conservation of this endangered species, this paper presents morphological aspects on the gametophyte development of *Dicksonia sellowiana* (Dicksoniaceae) by light microscopy and scanning electron microscopy. *Dicksonia sellowiana* spores were germinated in Morh's nutrient solution modified by Dyer (1979) under a 16-hour photoperiod at 23 ± 2 C. To determine the best substrate for gametophyte and sporophyte development, 30 days after spore sowing filamentous gametophytes were transferred to different substrates: soil rich in organic matter; coxim (coconut fiber); sterilized typic hapludult soil (distroferric red nitosoil); and sterilized typic hapludult soil (distroferric red nitosoil) with the addition of organic compost. The best system for *D. sellowiana* growth was the red soil with the addition of compost. Fifteen days after spore sowing in mineral solution, gametophytes were filamentous. Some had attained laminar morphology and had established an oblique cell division, giving rise to the obconic cell. Laminar gametophytes were observed 30 days after spore sowing and cordate gametophytes were observed after 45 days. Mature cordate gametophytes were observed after 80–90 days. After 245 days 84.67% of gametophytes had produced sporophytes in sterilized red soil with the addition of organic compost. In typic hapludult soil, without the additional termophilic compost, sporophyte formation was delayed (development after 180 days). When gametophytes were transplanted to soil rich in organic matter they did not develop and in the "coxim" substrate, which is a substitute for the "xaxim" substrate, only filamentous gametophytes were observed at the end of the study.

Key Words.—*Dicksonia sellowiana*, gametophyte development, substrate comparison

The Atlantic Forest biome entirely occupies three Brazilian states: Espírito Santo, Rio de Janeiro and Santa Catarina, 98% of Paraná and some areas of 11 other states (IBGE, 2004; Fundação Biodiversitas, 2006). Ferns are an important plant group of the Brazilian flora. According to Tryon (1970, 1972) and Tryon and Tryon (1982), southeastern Brazil (from Minas Gerais to Rio Grande do Sul) contains about 600 fern species. Some of the Brazilian ferns are used as ornamental plants and members of the tree fern families Cyatheaceae, Dicksoniaceae and Cibotiaceae have been indiscriminately exploited through the commercialization of pots and substrate used in the production of ornamental plants (Windisch, 2002). For that reason, under-

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standing aspects of fern biology is necessary for the development of methods that may assist in their conservation and management.

In Brazil, *Dicksonia sellowiana* Hook. (Dicksoniaceae) is considered an endangered species of the Atlantic Forest biome (IBAMA, 1997). It occurs preferentially in high humidity environments and on river banks, independent of soil conditions (Fundação Biodiversitas, 2006). The stem is usually massive, ranging from 12–20 cm in diameter, arborescent, 10 m tall, basally decumbent, bearing long, dense trichomes and many fibrous roots, which may occur from the base almost to the apex. It occurs at ca. 1500–2500 m, sometimes up to 3500 m, or in Brazil at lower elevations. It occurs throughout Central America and in South America from Venezuela to Colombia, south of Bolivia, Paraguay, Uruguay and southeastern Brazil (Sehnem, 1978; Tryon 1970, 1972; Tryon and Tryon, 1982). In Brazil, it is known as 'xaxim' or 'xaxim bugio' and the trunks have been indiscriminately exploited through the commercialization of vases and substrate (Sehnem, 1978).

The understanding of fern germination and establishment is required for their "ex situ" conservation. The germination of a great number of fern spores is promoted by light (Millër, 1968) and nutrients, water and mild temperatures are implicated in the growth and development of the prothallus and in sporophyte formation (Fernández *et al.*, 1996, 1999). Several aspects of the germination of *D. sellowiana* have been studied. Fillipini *et al.* (1999) sterilized spores in a 5% solution of commercial bleach for 10 min and reported that the spores of this species reached around 88% germination at 23 ± 2°C under continuous white light, seven days after sowing in liquid mineral medium. The same authors reported that the spores stored under refrigeration remained viable for more than two years and reached 81.75% germination 10 days after spore sowing, which did not differ from the germination of recently collected spores (Fillipini *et al.*, 1999). Under 50% and 36% irradiance, the germination of *D. sellowiana* spores was delayed after 14 and 21 days, respectively, of culture compared to 20% and 5% irradiance. Higher percentages of germination (around 90%) and lower mean germination time (34 days) were observed for spores of *D. sellowiana* sterilized in a 35% solution of commercial bleach for one hour, which germinated at 20% and 5% sunlight; no statistically significant differences were observed between the two light treatments (Fillipini *et al.*, 1999; Renner and Randi, 2004). To study the possibility of long-term spore storage of *Dicksonia sellowiana* for the establishment of a germplasm bank, Rogge *et al.* (2000) stored spores in liquid nitrogen and reported that spores remained viable after being immersed in liquid nitrogen for three months. Concerning patterns of gametophyte development, Nayar and Kaur (1969) described seven different types of prothallial development in the homosporous ferns. In previous works it was reported that germination in *D. sellowiana* is of *Vittaria* type and prothallic development is of *Adiantum* type (Pérez-Garcia and Fraille, 1986).

To obtain sporophytes of *Dicksonia sellowiana* cultivated from germinated spores, Borelli *et al.* (1990) cultivated *D. sellowiana* in the soil of "xaxim" (*D. sellowiana*) trunks and observed sporophytes after six months of spore sowing.

They commented that fungal contamination was very high in all the treatments. Suzuki *et al.* (2005) cultivated *D. sellowiana* from spores and observed sporophytes that had been emerged in sterilized typic hapludult soil (red soil) with the addition of termophilic organic compost; the first sporophyte frond was observed 84 days after transplantation.

The aim of the present study was to observe gametophytes of *Dicksonia sellowiana* grown in different substrates in the laboratory to examine morphological aspects of gametophyte development using light microscopy and scanning electron microscopy in order to determine suitable conditions for their growth and development.

Material and Methods

Sporophylls of *Dicksonia sellowiana* were collected from living plants in August 1999 in Urupema, a fragment of the Atlantic Forest biome, situated between 27°57′25″S and 49°53′33″W, Santa Catarina state, Brazil. Sporophylls were air-dried in an oven at 30°C for three days on filter paper in order to induce dehiscence. The spores were removed and separated from debris by filtering through lens paper, and then were stored in glass jars under refrigeration at 7 ± 1°C.

Spores were surface-sterilized using a 20% (v/v) solution of commercial bleach (2% active chlorine), which corresponds to 0.1% of active chlorine, for a period of 30 min before filtering through sterile filter paper and washing several times with sterile distilled water. About 20 mg of sterilized spores were sown in each of 32 conical flasks containing 20 ml of Mohr's nutrient solution as presented by Dyer (1979) with the addition of 25 mg L^{-1} Benomyl to avoid fungal contamination. The flasks were plugged with two layers of autoclaved transparent commercial polypropylene film (7 × 7 cm) fixed with rubber bands. All the procedures were carried out in a laminar hood. The spores were incubated in a 16-hour photoperiod (30 μmoles quanta · m^2 · s^1) at 23 ± 2°C in January 2002.

In February 2002, the young gametophytes cultivated in liquid medium were transplanted to trays containing four types of substrates: commercial substrate rich in organic matter; commercial "coxim": substrate produced from the coconut fiber used as substitute for the soil made from the "xaxim" (*D. sellowiana* trunks); sterilized typic hapludult soil (distroferric red nitosoil) and sterilized typic hapludult soil (3 parts) with addition of termophilic organic compost (1 part) as described in Suzuki *et al.* (2005).

The soil analysis was carried out in Soil Laboratory of CIDASC (Company for Agricultural Development of Santa Catarina) (Table 1). The trays were covered with transparent film to avoid excessive water evaporation and plant dehydration. Substrate sterilization was carried out in a high power microwave oven for 20 minutes. The organic compost was produced from vegetable and fruit wastes at the University of Santa Catarina. Sporophyte emergence was scored once a week. The mean and standard deviation for each day of evaluation was calculated by Excel for Windows (Microsoft).

TABLE 1. Analysis of substratum mineral composition (CIDASC-analysis number 07462/2003).

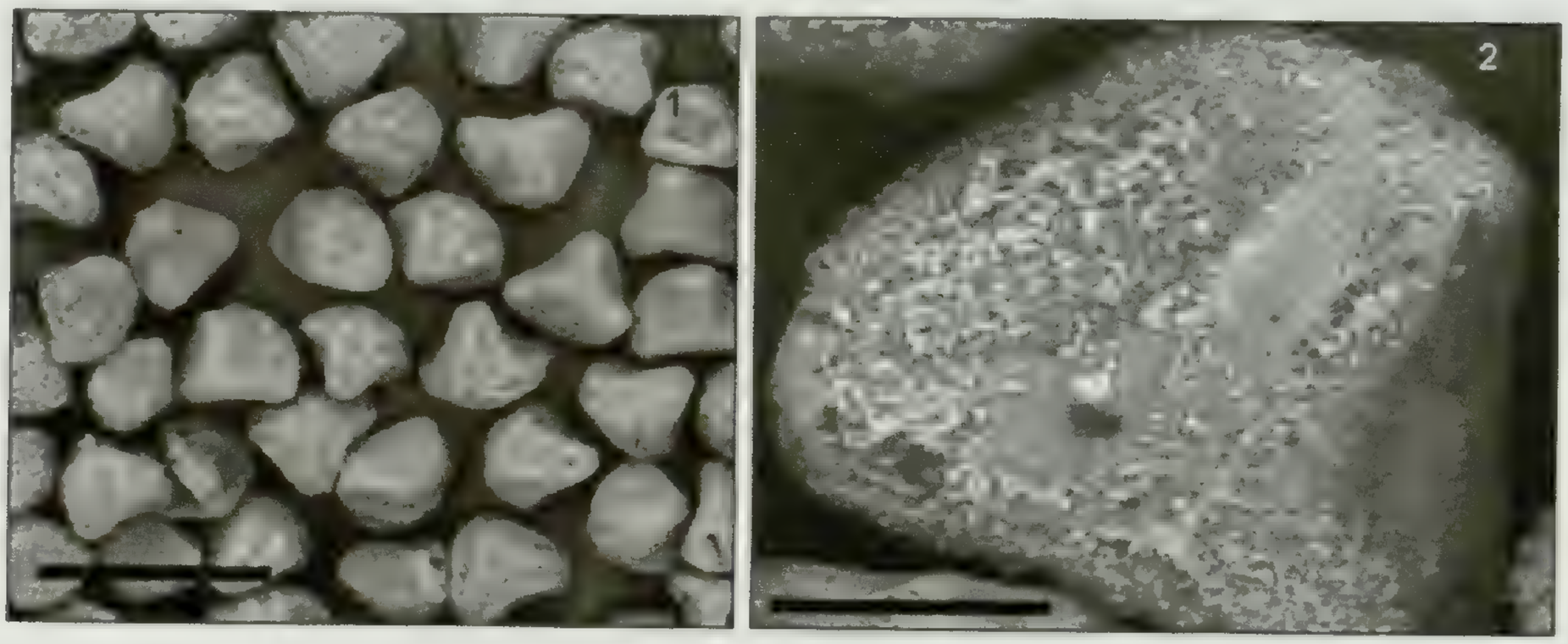
	Soil rich in organic matter	Coxim	Typic hapludult soil	Typic hapludult soil plus termophilic compost
pH	6.60	5.20	4.40	5.20
P (ppm)	> 50.00	38.30	2.60	> 50.00
K (ppm)	340.00	1204.00	95.00	450.00
% organic matter	4.30	> 10.00	0.80	0.90
% total N	0.12	0.35	0.03	0.26
Al (cmolc/l)	traces	0.3	2.2	traces
Ca (cmolc/l)	5.4	1.7	1.7	4.8
H^+, Al (cmolc/l)	2.48	1.89	8.79	3.90
CEC (cmolc/l)	13.92	9.37	11.32	12.88

Specimens were collected every 15 days from the mineral solution and from the trays containing sterilized typic hapludult soil and organic compost. For light microscopy (LM) and scanning electron microscopy (SEM), gametophytes were fixed in 2.5% glutaraldeyde in 0.1 M sodium phosphate buffer 7.2 pH. All samples were fixed in Eppendorf tubes for 3-hr, then were centrifuged for 3 min, dehydrated with an ethanol series and stored in ethanol. The samples were photographed with a Leika-MPS 30 light microscope. For LM, the samples were mounted on glass slides with ethanol. For SEM, the cordate gametophytes were dehydrated with graded ethanol (80%, 90%, 96% and 3 times in 100%). Subsequently, they were transferred to HMDS (hexamethyldesilasane) to substitute the CO_2 critical point, avoiding cell collapse (Bozzola and Russel, 1991). Dry samples were transferred to stubs and then were gold-coated with 20 nm of gold in a Baltec-CED 030. Examination was performed with a Philips-XL 30 scanning electron microscope.

RESULTS

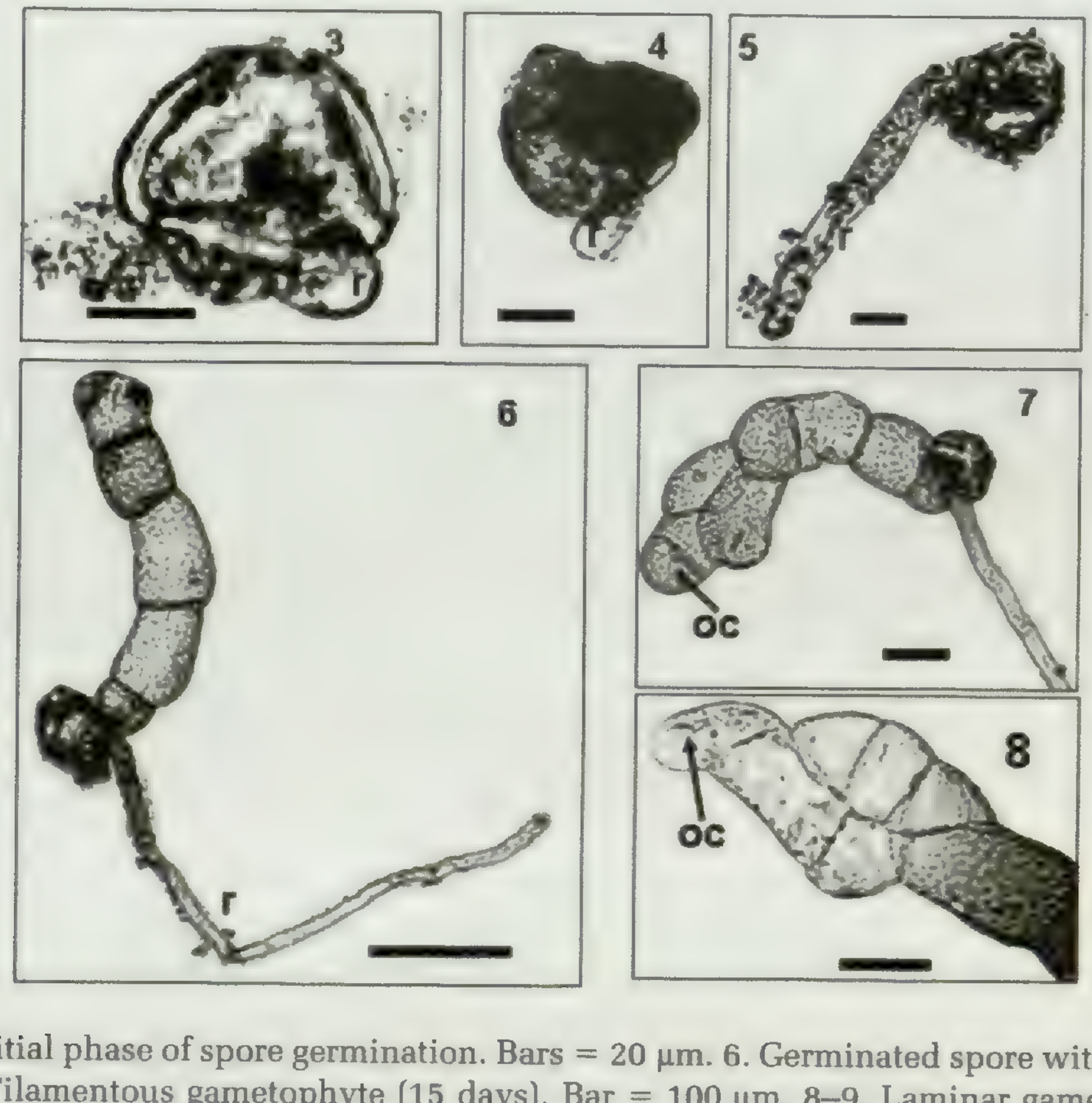
The spores of *Dicksonia sellowiana* are tetrahedral, globose and trilete; the surface is densely granulated and measure 44–68μm (Figs. 1 and 2). *Dicksonia sellowiana* germination is of the *Vittaria* type (Pérez-Garcia and Fraille, 1986). During germination, the spores become swollen and the spore coat opens. The first division was parallel to the equatorial axis of the spores; small hemispheric cells were produced and gave rise to a hyaline rhizoid that does not contain plastids; subsequently a spherical prothallial cell which is rich in chloroplasts appeared (Figs. 3–5).

Gametophytes of *D. sellowiana* were not able to develop in the soil rich in organic matter. In the coxim, only filamentous gametophytes were observed after 8 months of cultivation. In sterilized typic hapludult soil, the first sporophytes were only observed after 6 months of cultivation, but in sterilized typic hapludult soil with addition of termophilic organic compost, sporophytes were observed after less than 3 months of cultivation.

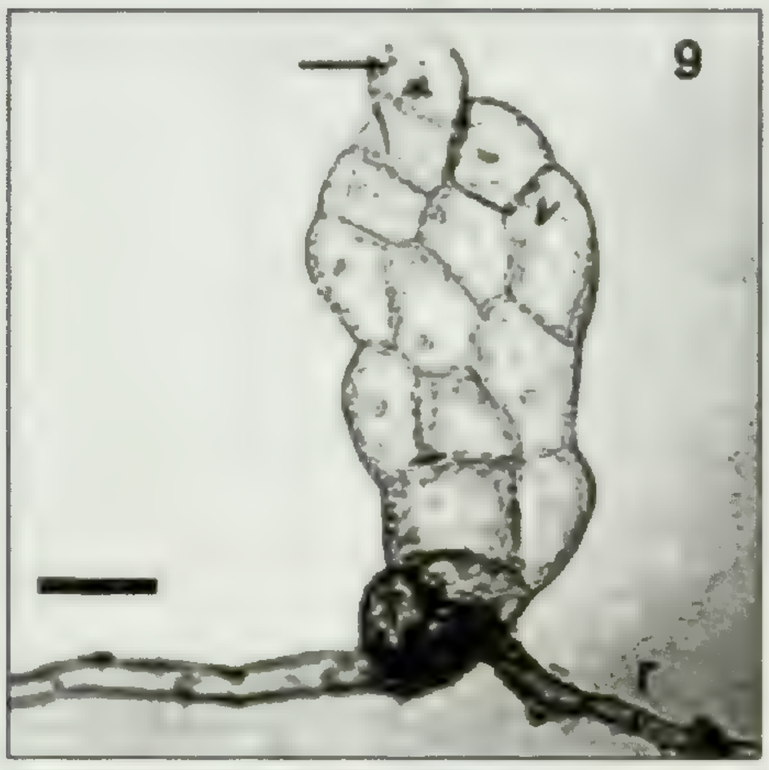


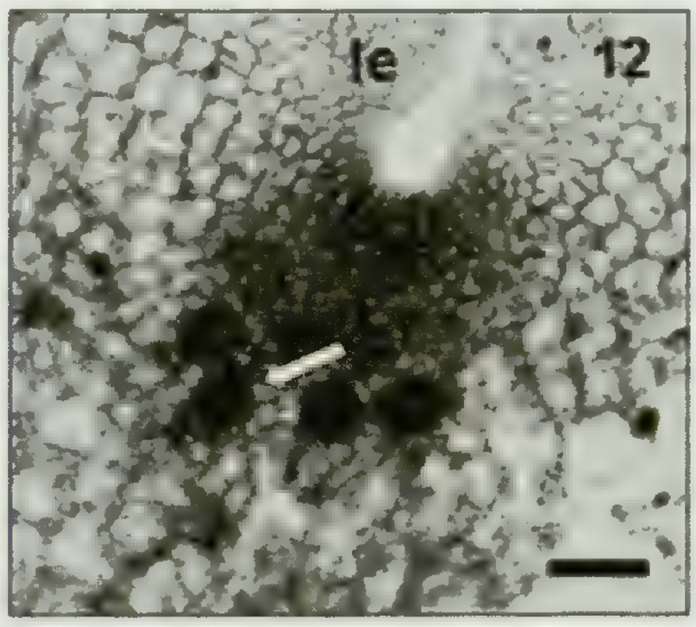
FIGS. 1–2. 1. Scanning electron micrographs of *Dicksonia sellowiana* spores. 2. Tetrahedral spores. Bar = 100 μm. 3. Detail of the spore with densely granulated surface. Bar = 20 μm.

Fifteen days after sowing, a uniseriate filament was apparent, consisting of 3–7 cells as a result of parallel divisions of the original prothallial cell. The filament cells showed abundant chloroplasts and the spore coat was still attached to the basal cell. There was only one rhizoid present (Fig. 6). A wall parallel to the axis of the filament divided the terminal cell; further division followed an oblique direction, giving rise to an obconic or meristematic cell (Figs. 7 and 8). The laminar phase of *D. sellowiana* gametophytes was observed after 30 days of spore sowing. The beginning of the cordate or heart phase with



FIGS. 3–8. Initial phase of spore germination. Bars = 20 μm. 6. Germinated spore with rhizoid. Bar = 20 μm. 7. Filamentous gametophyte (15 days). Bar = 100 μm. 8–9. Laminar gametophyte with the initial lateral divisions (15 days). Bars = 50 μm. Legend: oc – obconic cell, r - rhizoid.



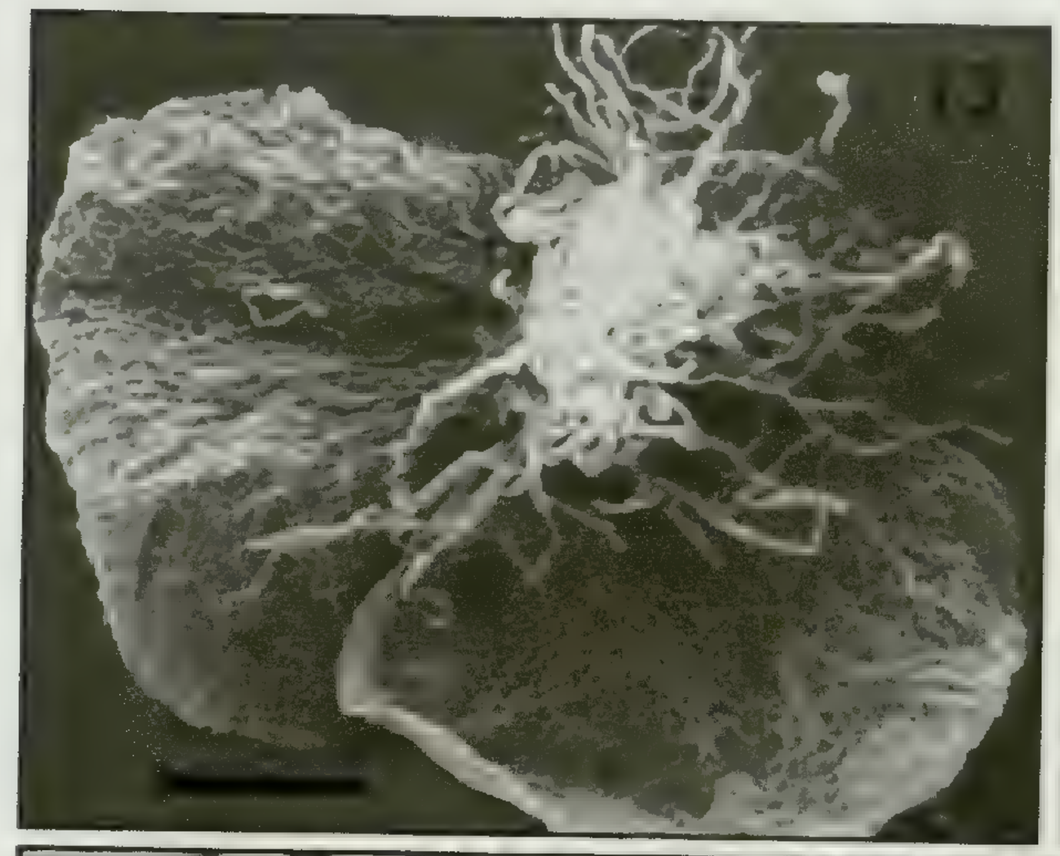


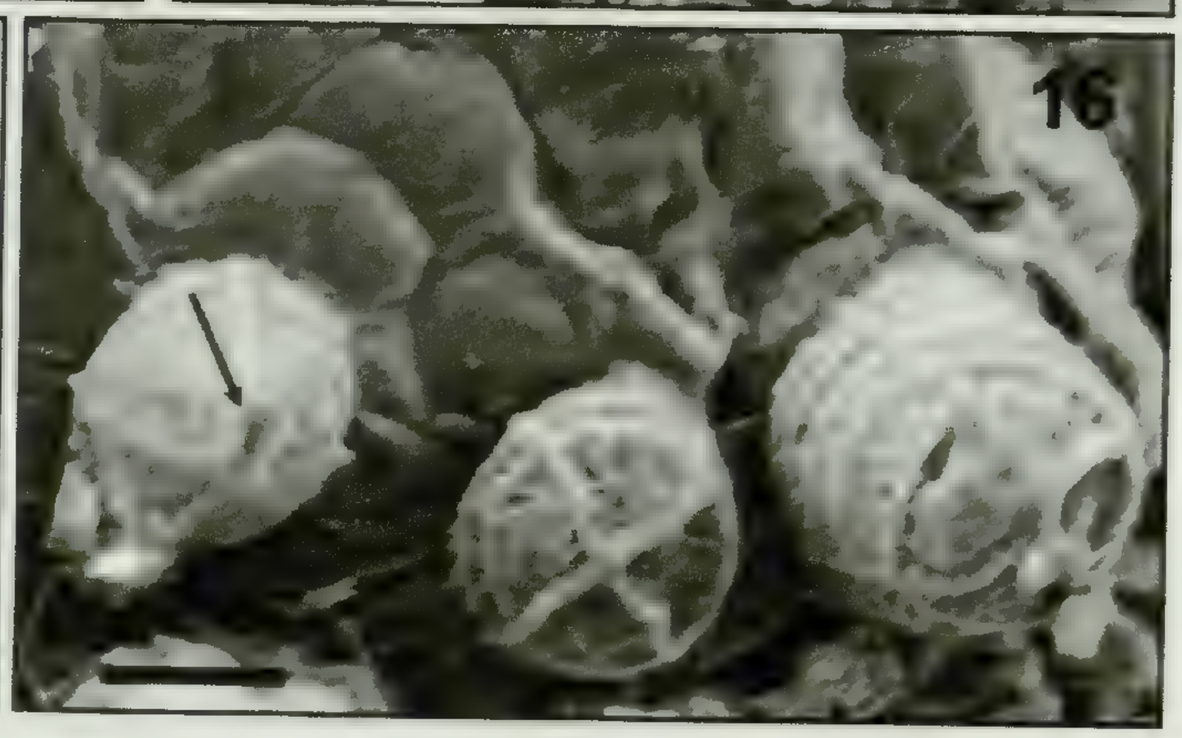
FIGS. 9–12. 9. Laminar phase (30 days) Bar = 50 μm. 10. Gametophyte spatulated (45 days). Bar = 100 μm. 11. Cordate gametophyte (75 days). Bar = 200 μm. 12. Meristematic region with archegonia (90 days). Bar = 100 μm. ac- obconic cell , amr - apical meristematic region,– ar- archegonium, le - lateral expansion, r – rhizoid.

the development of the wings was observed 45 days after spore sowing and 15 days after gametophyte transplantation to the sterilized typic hapludult soil with addition of termophilic organic compost. After 75 days of spore sowing, gametophytes presented the heart shape (Figs. 9–11) and 90 days after spore sowing archegonia were present (Figs. 12 and 13). The archegonia grew from surface cells of the apical meristem, were bottle-shaped, and presented four rows of 4–5 exposed neck cells and the neck canal inside (Figs. 13–16). Antheridia were not observed in this SEM prepared material. The gametophytes did not bear trichomes.

DISCUSSION

The surface *of Dicksonia sellowiana* spores are granulated or reticulate with strands of fused spheres surrounding somewhat depressed areoles as observed by Tryon and Tryon (1982). The pattern of spore germination found for *Dicksonia sellowiana* was the *Vittaria* type (Nayar and Kaur 1971), which was described by Pérez-Garcia and Fraille (1986). The prothallial development of *Dicksonia sellowiana* is of the *Adiantum* type, described by Nayar and Kaur





FIGS. 13–16. Scanning electron micrographs of *Dicksonia sellowiana* gametophytes. 13. General gametophyte view. Bar = 500 μm. 14. Aspects of inferior face bearing archegonia. Bar = 100 μm. 15. Detail of an archegonia. Bar = 20 μm.

(1969) and Pérez-Garcia and Fraille (1986). In this type of gametophyte development, spore germination results in a uniseriate, slender germ filament, which is generally 3–7 cells long. The terminal cell and one or two cells behind it divide longitudinally to form a prothallial plate. The division of the terminal cell is often by the formation of a wall oblique to the long axis of the filamentous gametophyte, and soon a second oblique wall delimits a central obconical meristematic cell. By the activity of the obconical cell, a spatulated prothallial plate, without trichomes, is formed in which the apex gradually becomes notched. Examples of species from the Dicksoniaceae that show the *Adiantum* type protallial development are *Lophosoria quadripinnata* (J. F. Gmel) C. Chr. (Pérez-García *et al.*, 1995) and *Lophosoria quadripinnata* var.*contracta* (Mendoza *et al.*, 1997).

The laminar phase was observed after 30 days of cultivation for *Dicksonia sellowiana* and the heart shaped gametophytes presented archegonia 90 days after spore sowing, but they did not present antheridia. Conversely, Pérez-Garcia and Fraille (1986) observed gametophytes of *D. sellowiana* bearing antheridia only after 170 days cultivation, but they did not observe gametophytes bearing archegonia. The same authors analyzed gametophyte development only under light microscopy and did not analyze time to

sporophyte formation and percentage of sporophyte formation in different soils. They found filamentous amorphous gametophytes that were not observed in the present work. The gametophyte of *Lophosoria quadripinnata* was spatulate after 156 days of culture; antheridia were observed after 72 days, but archegonia were seen only after 270–285 days (Pérez-García *et al.*, 1995). Additional analyses are necessary to show how sexual expression of *D. sellowiana* is affected by spore density in laboratory conditions.

In Santa Catarina State (Brazil) there is a predominance of clay soils, cambisoils, laterites and nitosoils (IBGE, 2005). In the experimental conditions carried out in this study, the typic hapludult soil (distroferric red nitosoil) with the addition of termophilic organic compost was the most suitable for *Dicksonia sellowiana* development, and the first sporophytes in this substrate were observed 84 days after spore sowing. Atlantic Forest soils are poor in nutrients and the accumulation of chemical elements in cells is one of the ways tropical species tolerate low-nutrient soil (IBGE 2004; Fundação Biodiversitas, 2006). Litterfall is a fundamental component of nutrient cycling, and it is the main means of transferring organic matter and mineral elements back to the soil surface (De França *et al.*, 2007; Moraes *et al.*, 1999). The termophilic organic compost was added in order to simulate the litter in this substrate. This substrate has a low pH and high levels of N, P, K and Ca. In contrast, when Borelli *et al.* (1990) cultivated gametophytes of *Dicksonia sellowiana* in the soil made of "xaxim" trunks, the first sporophytes were observed only after six months of cultivation and the authors observed 100% contamination in their cultures and around 75% of gametophytes produced sporophytes. Therefore, the time to sporophyte formation, the percentage of sporophyte formation, and the prevention of contamination were improved in the present work. Edaphic parameters were analyzed for several fern species to elucidate their habitats including nutritional requirements and the majority of them prefer acidic soils, as does *D. sellowiana* (Carlson, 1979; Graves and Monk, 1982; Whitter and Moyroud, 1993; Ranal, 1995). The time to sporophyte emergence is quite variable among fern species; *Lophosoria quadripinnata* formed sporophytes after 36 months cultivation (Pérez-García *et al.*, 1995).

The typic hapludult soil with added termophilic organic compost employed in this work was also useful for sporophyte emergence, which was observed less than three months after cultivation as observed in previous work (Suzuki *et al.*, 2005).

Information provided in this paper certainly will be useful for *D. sellowiana* management in green houses as part of conservation strategies. Plantlets of *Dicksonia sellowiana* can be easily obtained following the protocol presented here. This methodology can be used for the establishment of germplasm banks with the purpose of preserving the species in botanical gardens and to maintain its genetic variability.

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In vitro Study on Gametophyte Development of an Epiphytic Fern, *Arthromeris himalayensis* (Hook.) Ching, of South Sikkim, India

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ABSTRACT.—Gametophyte development in *Arthromeris himalayensis* was studied and found to be of "*Drynaria* type". Germination occurred 9–10 days after sowing of spores. Some prothalli showed an initial archegonial phase, which persisted throughout gametophyte development and the antheridial phase developed on separate thalli a few days later and persisted throughout the life span of the gametophytes. This type of development of sex organs may be considered as a variant new type from the previously reported types by earlier authors. This variant type is described here as "type H". This type of gametangial development on separate prothalli is an indication of adaptation for out breeding.

KEY WORDS.—Gametophyte development, *Arthromeris himalayensis*, Epiphyte, Type H, out-breeding

Arthromeris himalayensis (Hook.) Ching belongs to the family Polypodiaceae and is a warm temperate fern, exclusively epiphytic in nature and distributed in India throughout the Himalayan region from Eastern Himalayas to Western Himalayas. This epiphytic fern is also found in China, Nepal and Burma in mountain areas. In Southern Sikkim this fern is generally found between 2700–3600 m.

Germination of fern spores, growth, and further development of resulting gametophytes in artificial media is a well-studied area in pteridophyte and developmental biological research (Nayar, 1962; Atkinson and Stokey, 1964; Kato, 1969; Klekowski, 1969; Nayar and Kaur, 1969; Nayar and Kaur, 1971; Masuyama, 1975a, b; Khare and Kaur, 1983; Raghavan, 1989; Chiou and Farrar, 1997; Verma *et al.*, 2000; Verma, 2003; Ganguly and Mukhopadhyay, 2005). Nayar and Kaur (1971) and Atkinson (1973) pointed out that the sequence and plane of cell divisions, pattern of gametophyte development, as well as the direction of initial growth of the first rhizoid and germ filament with respect to the polarity of germinating spore are distinct characteristics and can be utilized effectively for drawing phylogenetic relationships among various taxonomic groups. Nayar (1962) studied the spore germination and prothallial morphology of *Arthromeris wallichiana* (Sprengel) Ching along with some other polypodiaceous ferns. However, no work has been done on the prothallial development of the epiphytic fern *Arthromeris himalayensis*. Ferns

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occupy a specialized habitat as epiphytes and, as such, epiphytic ferns have evolved various gametophytic generation adapations like antheridiogen systems, production of gemmae, indefinite growth of prothalli, etc. (Farrar, 1974, 2003). Among the 15 species of *Arthromeris* distributed throughout the world, the majority (10 species) are found in the Eastern Himalayas (Ghosh *et al.*, 2004). Out of these 10 species, *Arthromeris himalayensis* grows very successfully in the highest altitudes as epiphytes and has high antimicrobial activity (Ganguly *et al.*, 2008). These interesting attributes prompted us to study its reproductive behavior and gametophyte development. The current study was performed to understand the details of gametophyte structure and development pattern of prothalli in *Arthromeris himalayensis*.

Materials and Methods

Mature sporophylls of *Arthromeris himalayensis* were collected from healthy plants from Maenum Wildlife Sanctuary (3023 m altitude), South Sikkim in the November of 2005 and 2006. Sporophylls of individual plants were kept separately within blotting paper and mature spores from sporophyll(s) of individual plants dehisced within 48 hrs were collected in separate vials for gametophyte studies. Collected spores from three individual sporophytes were sown in separate petri plates on modified Moore's medium (Kato, 1969). Two replicates of each set were maintained. These spores were surface sterilized by 0.1% $HgCl_2$ (w/v) solution for 5–8 minutes and rinsed three times with sterilized distilled water and then dried on sterilized blotting paper. The sterilized spores were transferred to autoclaved (at 15 lb/inch^2 for 15 minutes) modified Moore's culture medium (Kato, 1969) solidified by 1% (w/v) agar in an aseptic chamber and the pH of the medium was maintained at 5.8. The cultures were incubated at 22°C–25°C under cool fluorescent white light (ca 1000 lux, 16hr/d).

Gametophytes from each petri plate were studied every day randomly by light microscope (Leica DMLB) after germination of spores. Time taken for spore germination and to form mature gametophytes, initiation of sex organs and formation of sporophytes were recorded. Camera Lucida drawings of different developmental stages were made on the same microscope. Records of gametophyte development patterns from individual sporophytes were maintained separately in order to see if there were variations in the developmental patterns among the individuals. Observations were made on ten gametophytes from each petri plate at a time.

Results

Characteristics of spore germination and gametophyte development.— Spores were bilateral, monolete; light brown in color, perisporate, perispore thin, size 38–42 × 50–53 μm. Spore germination of *Arthromeris himalayensis* was 79.49 ± 4.66%. Spores germinated even after having been stored for two months after collection.

Spores germinated 9–10 days after sowing. In this species the rhizoidal cell formed first (Fig. 1A) followed by the chlorophyllous protonemal cell (Fig. 1B). The protonemal cell developed into a 6-celled stage by periclinal divisions (Fig. 1C–E). Spore germination resulted in a slender uniseriate germ filament. The penultimate protonemal cell underwent oblique vertical division. In *Arthromeris himalayensis* the establishment of an apical meristem was much delayed and the prothalli usually developed hairs on the margin and surfaces. A broad spathulate prothalli plate was formed by repeated longitudinal and transverse divisions of its anterior cells and expansion of the resultant daughter cells (Fig. 1F–K). Mature vegetative, cordate shaped gametophytes (Fig. 1M) developed 77–80 days after spore germination. The mature prothalli measured ca 350 × 300 μm in size. The prothallial plate often became 15–20 cells or more wide and broadly ovate, but was devoid of any organized meristem. Later, an obconical meristematic cell was differentiated by two oblique divisions in one of the marginal cells at the anterior end of the prothallial plate (Fig. 1K). The meristematic region (Fig. 1L–M) was located under notch. The type of development was purely "*Drynaria* type" as discussed by Nayar and Kaur (1969, 1971).

Development of sex organs (sequence, position and duration).—Mature cordate gametophytes remained vegetative for about 30 days, after which the gametophytes started to develop sex organs. Archegonia developed first in some cordate shaped prothalli 112 ± 2 days after spore germination. Archegonia were situated along the midrib region and just below the meristematic region. Archegonia consisted of a projecting neck (Fig. 1M) and a lower embedded venter. This flask shaped structure was made up of two axial rows of neck canal cells, one ventral canal cell and one egg cell (Fig. 1N). Each archegonium had a single layered jacket (Fig. 1N) and was 150–200 × 65–75μm in size. Antheridia developed on separate gametophytic prothalli, which were elongated and much longer than archegonial prothalli. Initiation of antheridia started 115 ± 2 days after spore germination. Antheridia were of the emergent type (Fig. 1O) with a 1-cell thick jacket, measuring about 25–30μm.

In *Arthromeris himalayensis*, the prothalli were dioecious. The cordate shaped prothalli developed archegonia after they reached maturity and remained as archegoniate prothalli throughout the reproductive phase. Antheridiate prothalli were elongated and did not form well-defined apical meristem. Antheridia developed on the lower half of the prothallus, marginal and/or superficial in position (Fig. 1L). After initiation, antheridia took about 3–5 days to mature; spermatozoids were released after this period. The time taken for development of the different gametophytic stages of *Arthromeris himalayensis* is shown in Table 1.

Discussion

From the above observations, we can conclude that the type of gametophyte development in *Arthromeris himalayensis* is purely "*Drynaria* type". In

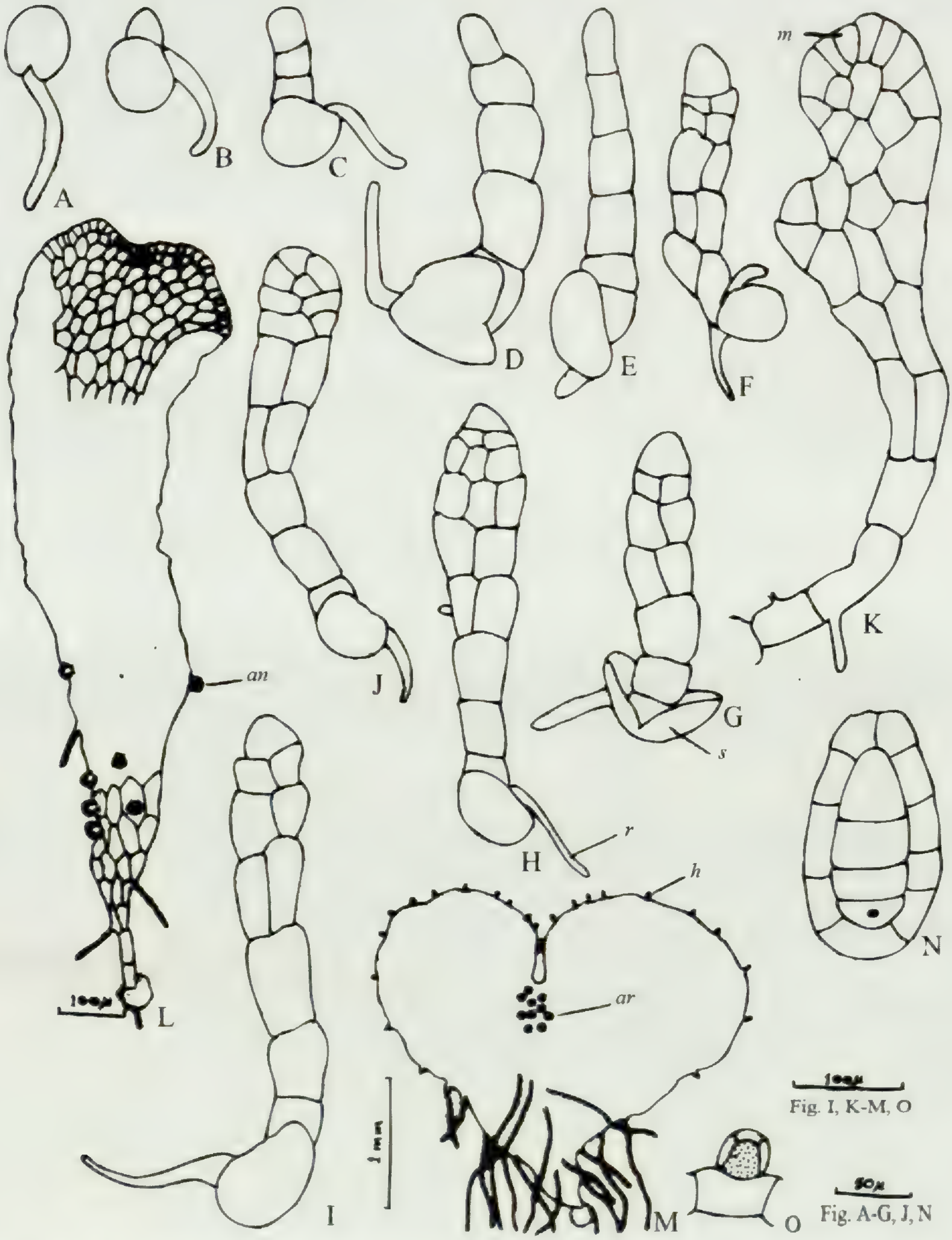

Fig. I, K-M, O

Fig. A-G, J, N

FIG. 1. **A–O.** Different stages of gametophyte development of *Arthromeris himalayensis*. **A.** Germination of spore showing first rhizoidal cell. **B.** Emergence of first prothallial cell. **C–E.** Different stages of filamentous prothallus. **F–J.** Stages in development of non-meristematic prothallial plate. **K.** Establishment of apical meristematic cell. **L.** Development of antheridia on antheridiate prothallus. **M.** Development of archegonia on mature cordate prothallus. **N.** A mature archegonium. **O.** A mature antheridium. **NB**:[*h* = Hair ; *m* = Obconical meristematic cell ; *r* = Rhizoid ; *s* = Spore coat ; *a* = Antheridium ; *ar* = archegonium].

TABLE 1. Time taken for gametophyte development of *Arthromeris himalyensis* (Hook.) Ching.

Sl. No.	Events of the gametophyte development	Total no. of days taken after sowing of spore ± SD
1.	Sowing of spores	0 ± 0
2.	Spore germination	10 ± 1
3.	Formation of mature cordate prothallus	80 ± 3
4.	Initiation of archegonia	112 ± 2
5.	Initiation of antheridia	115 ± 3
6.	Maturation of antheridia	118 ± 3
7.	Initiation of sporophyte	123 ± 2

"*Drynaria* type" development, spore germination results in a slender uniseriate germ filament. A broad spathulate prothallial plate is formed by repeated longitudinal and transverse divisions of its anterior cell and expansion of the resultant daughter cells. The prothallial plate often becomes 5–10 cells or more wide and broadly ovate, but is devoid of any organized meristem. Later, an obconical meristematic cell is differentiated by two oblique divisions on one of the marginal cells at the anterior end of the prothallial

TABLE 2. Classification of gametangial sequence on meristematic prothalli of homosporous ferns (adapted from Verma 1989, 2003).

Sequential bearing of gametangia on meristematic prothalli			
Type	Initial state	Final state	Symbol
A	Antheridiate	Archegoniate.	M → F
	Archegoniate	Persists throughout.	F → F
B	Antheridiate	Antheridia and Archegonia formation.	M → H
	Archegoniate	Antheridia and archegonia formation.	F → H
C	Antheridiate	Antheridia and archegonia formation for some time, then only archegonia formation.	M → H → F F → H → F
	Archegoniate	Same	
D	Antheridiate	Antheridia and archegonia formation for some time, alternating periodicity in the formation of antheridia and archegonia, finally hermaphrodite.	M ↔ H ↔ F ↔ H F ↔ H ↔ M ↔ H
	Archegoniate	Same	
E	Antheridiate	Archegonia formation.	M → F
	Archegoniate	Antheridia and archegonia formation.	F → H
F	Archegoniate	Antheridia and archegonia formation (ephemeral), then antheridia formation.	F → H → M or F → M
G	Archegoniate	Antheridia and archegonia formation simultaneously.	F → H
H*	**Archegoniate**	**Persists throughout.**	**F → F**
	Antheridiate	**Persists throughout.**	**M → M**

Symbols indicate the sequential state of functional sex:
M = Antheridia formation, F = Archegonia formation, H = Hermaphrodite.
Types A, B and C are according to Masuyama (1975 b). Type D, E and F are proposed by Verma (1989). Type G is proposed by Ganguly & Mukhopadhyay (2005). Type **H*** is a new variant type proposed here by current authors.

plate. The young prothallus becomes cordate, the apical meristematic cell is replaced by a pluricellular meristem and a midrib developed. Young prothalli are naked; hairs are usually formed when the prothallial plate becomes cordate (Nayar and Kaur, 1969). The *Drynaria* type of development is characteristic of Cheiropleuriaceae, Dipteridaceae, Gleicheniaceae, Lomariopsidaceae, Loxomaceae, Thelypteridaceae and the majority of the Polypodiaceae genera (Nayar and Kaur, 1969). Smith *et al.* (2006) did not consider Cheiropleuriaceae a separate family; they merged the genus *Cheiropleuria* in the family Dipteridaceae. According to Masuyama's (1975a, b) classification of gametophytes, based on gametangial sequence of development on meristematic prothalli, which was further elaborated upon by Verma (1989, 2003) and Ganguly and Mukhopadhyay (2005), the gametophytes of *Arthromeris himalayensis* resemble 'type A' to some extent. In type A, the archegoniate prothalli persist throughout development but the antheridiate prothalli become archegoniate in the later stages. In *Arthromeris himalayensis*, the sequence of development of the sex organs is different. Here, the archegoniate prothalli remain archegoniate and the antheridiate prothalli remain antheridiate throughout development. Based on the sequence of sex organ development in *Arthromeris himalayensis*, it is identified as a new type, different from the types described by Masuyama (1975a, b), Verma (2003), and Ganguly and Mukhopadhyay (2005). Thus, we propose a new type "Type H" in addition to the existing seven types classified by the previous authors (Table 2).

Gametophyte growth habit can be classified into three basic types in regard to the effect of form on breeding system. Type I is the familiar cordate or butterfly shaped gametophytes of most terrestrial ferns. Type II gametophytes have indeterminate growth and branching and type III gametophytes combine type II growth with production of dispersible gemmae. Type II and type III gametophytes are typical of most epiphytic species (Farrar, 2003). In *Arthromeris himalayensis*, the archegoniate prothalli resemble type I, which is cordate shaped. The antheridiate prothallus was elongated, having indefinite growth. Some of the gametophytes showed clonal elongation. The secondary gametophytes produced antheridia on their margins. Thus, antheridiate prothalli resemble type II gametophytes partially.

The gametophytes of *Arthromeris himalayensis* are long lived (more than 110 days), and the advantage of long-lived gametophytic generation is to promote cross-fertilization (Klekowski, 1973, 1979). Opportunities for gamete exchange between long-lived gametophytes are much higher than for short-lived, non-clonal epiphytic gametophytes.

Most species of Polypodiaceae maintain an antheridiogen system through which the robustly growing female gametophytes induce production of antheridia precociously on the smaller gametophytes growing nearby, thus enhancing the probability of cross-fertilization (Chiou and Farrar, 1997). *Arthromeris himalayensis* may have an antheridiogen system, as antheridia grow on separate prothalli after 3–5 days of initiation of archegonia in cordate

shaped prothalli. This suggests that antheridiogen might have some role in controlling the reproductive system of *A. himalayensis*.

Masuyama (1975b) recognized four basic locations of antheridia on monoecious prothalli: antheridia on the lower part of gametophyte thallus (type L), on the lower half of the wings (LW), on the lower half of the margin (type LM), on the upper half of the central cushion (type UC), or antheridia located all along the margin (type M). As *Arthromeris himalayensis* produces dioecious prothalli, it does not resemble any type as recognized by Masuyama (1975b), though the antheridia located on the lower half of the wings (type LW) as proposed by Masuyama (1975b).

It is interesting to note that the percentage of spore germination is very high; about 79.49 ± 4.66% even after two months of harvesting. This figure indicates that this species produces a high proportion of viable spores, which is likely helpful in the survival of this species. This species is restricted to a certain altitudinal regions (2700–3600 m), thus specific environmental conditions like temperature, annual rainfall, relative humidity (RH), etc. are required for its survival. RH, annual rainfall and altitude have a combined effect on the distribution and reproductive success of this species (Ganguly and Mukhopadhyay, 2008).

Most homosporous fern gametophytes are potentially bisexual and due to continual self-fertilization there is a risk of exposing the lethal genes in homozygous condition. The gametophytic generation has evolved some adaptations to overcome this problem that influence the change of the mating system from intragametophytic to intergametopytic. These adaptations include the gender of the gametophytes, ecology, distribution and duration of gametangia on monoecious prothalli, and longevity of gametophytes and the capacity for vegetative reproduction (Klekowski, 1969; Lloyd, 1974a, b; Masuyama 1975a, b; Soltis and Soltis, 1987). From the above discussions, it may be concluded the *Arthromeris himalayensis* gametophytic generation shows some derived developmental features: 1) dioecious prothalli promotes intergametophytic fertilization (may be of sibling and/or non-sibling mating); 2) archegoniate prothalli that are meristematic and cordate shaped, continuously producing archegonia, increase the chances of sporophyte production; and 3) long-lived gametophytes (more than 110 days) that also promote intergametophytic fertilization.

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An Efficient Method for Surface Sterilization and Sowing Fern Spores *in vitro*

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Abstract.—Spores are commonly used to start *in vitro* culture of ferns. Numerous methods for spore surface sterilization and sowing have been developed, but spore loss and contamination are still problematic. To overcome these problems, an efficient method for sterilizing and sowing spores was established. Through this method, contamination and loss of spores is minimized, and can be sown in adjustable, even densities.

Key Words.—surface sterilization, *in vitro* culture, spore sowing

Surface sterilization is the first step for aseptic culture of ferns from spores (Dyer, 1979). Since tissue culture is widely employed as a technique for fern propagation or scientific studies, spores are widely used as a starting material. In recent years, there have been numerous studies on *in vitro* culture of ferns from spores (Stone, 1958; Yoroi, 1972; Kiss and Kiss, 1998; Cox *et al.*, 2003). These researchers reported successful cultures, but admitted significant losses of spores during the sterilization process, aseptic sowing, or due to contamination (Warne *et al.*, 1986). Because of these problems, different methods of spore sterilization and sowing have been developed (Dyer, 1979). Many of these methods are still inefficient in terms of time and spore loss (Warne *et al.*, 1986).

Recently, we established an effective method for sterilization of spores with a filter funnel. The method is successful with spores of *Osmunda japonica* Thunb., *Aleuritopteris argentea* Gmel., *Adiantum flabellulatum* L., *Adiantum capillis-veneris* L. and *Cyrtomium fortunei* J. Smith (data not shown). We compared our method, here called the filter method, with two other widely used methods. With the packet method, spores were sterilized in filter bags/packets (Ford and Fay, 1999), while with the centrifugation method the spores were suspended in a sterilizing solution and then harvested in sterile distilled water by centrifugation (Fernández *et al.*, 1993). For these comparisons, we used spores of *Adiantam reniforme* var. *sinense* Y. X. Lin, a rare and endemic species in China.

Materials and Methods

Plant materials.—Sporophytes of *A. reniforme* var. *sinense* were introduced from Wanxian County along the Yangtze River in 2001 and cultivated in the

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greenhouse of Huazhong Agricultural University (Wuhan, China). Fertile fronds of sporophytes in the greenhouse were collected and wrapped in paper bags and dried at room temperature for one week to release spores. Then the spores were collected in centrifuge tubes and stored at 4°C until used. For this study, spores were stored for 10 months.

Culture media.—*Murashige* and Skoog (1962) medium (MS) with 1/4 strength of macronutrients were used for germination of spores. The medium was supplemented with 3% (w/v) of sucrose, solidified with 0.65% (w/v) agar and adjusted to pH 5.8 before autoclaving at 121°C and 1.1 kg cm^{-2} for 20 min.

Spore sterilization and sowing.—The filter method was performed in a laminar flow hood. Three mg spores were suspended and wetted with 4% (v/v) Tween solution for 5 min in a 1.5 ml centrifuge tube. Suspended spores were collected through a filter funnel (made by fast filter paper), which was placed on a proper conical flask. The tube was washed three times with fresh water and the water was poured into the funnel to collect the residual spores of the tube. Seventy percent (v/v) alcohol (chemical purity) was added to immerge the spores along the funnel margin. Thirty seconds later 30 ml fresh sterile distilled water was continually added to rinse the spores.

After removing the alcohol, 4% (w/v) sodium hypochlorite (NaClO) or 0.1% (w/v) mercuric chloride ($HgCl_2$) was added along the funnel margin. For different disinfectants, the sterilizing time was different: NaClO (5–6 min) and $HgCl_2$ (2–3min). At the end of the sterilizing time, fresh sterile distilled water was full filled into the funnel along the margin, regardless of whether the $HgCl_2$ or NaClO solution had completely drained off or not. The same operations were repeated twice when the dilute solution seeped half from the filter funnel. Then after the solution drained off, spores were thoroughly rinsed six times with sterile distilled water. After the sterile distilled water drained off, the spores were rinsed with 40 ml fresh sterile distilled water from the filter paper into a sterile container. Using a sterile pipette, the spore suspension was distributed onto culture plates (9 cm diameter Petri dishes, containing 20 ml culture medium), which were sealed with plastic film. The sterile distilled water and different solutions were transferred by transfer pipette with sterilized tips (1ml).

Ten plates were made for each treatment and the experiment was conducted three times. The waste of the $HgCl_2$ solution was collected and adjusted to pH 8–10. Enough Na_2S and $FeSO_4$ were then added to react with the $HgCl_2$ and produce sediments. After sediments formed thoroughly, the sediments were collected and sent to the hazardous waste disposal department for detoxification and proper disposal.

All cultures were incubated in a controlled environment room that was maintained at 23±2°C under a light intensity of 25 μl m^{-2} s^{-1} with 16/8 photoperiod.

The spore density of one drop of spore suspension solution from a 1 ml transfer pipette in the filter method and centrifugation method were recorded and means with standard deviations (S.D.) were calculated. In the packet

method, spores were sown by directly wiping the spores on the medium, thus spore density was not scored. Contamination and spore germination were examined on the 40th day after spore sowing. For germination rates, at least 300 random spores per plate were scored.

For comparison to the other two methods, the published methodologies (Fernández *et al.*, 1993; Ford and Fay, 1999) were followed using 3 mg of spores, and are not described in this paper.

Sterilization capacities of different sterilization methods:

To test the sterilization capacities of the different methods, comparisons were made between different methods using different weights of spores (0.5g, 1.5g and 3g). The spores were administered into proper centrifuge tubes or packed in proper filter papers. All spores were sown in soil after sterilization.

Results

In the filter method, spores sterilized by NaClO were obviously bleached and it was difficult to judge whether the spores were rinsed completely from the filter paper or if spores drifted from the tubes when poured out of the disinfecting solutions and rinse water. Table 1 shows that there were no significant differences in spore density between the two disinfectants. However, the spore densities varied greatly between the different methods tested. From the centrifugation method, there were about 69–75 spores /drop, from the filter method, there were about 235–248 spores/drop, and the number of spores not sterilized and suspended in 40 ml water directly was 260–272 spores/drop (Table 1). In the packet method, sowing of spores was achieved by wiping the spores in a swirling motion over the surface of culture medium directly, so the spore density through this method was very high in the first plate and very low in the last plate. Nevertheless, the spores tended to clump together on the first plate. Thus, the spore densities changed greatly from plate to plate and across plates. Within a single plate, the highest density was more than 3000 spores cm^{-2}; the lowest density was less than 10 spores cm^{-2}.

The spores from the filter method and packet method started to germinate 10 days after sowing, while the spores from the centrifugation method started to germinate 15 days after sowing. On the 40th day, the highest germination (62.6%; see Table 1) was obtained from the filter method with $HgCl_2$, which was followed by the filter method with NaClO (60.8%, Table 1).

The germination rate of the packet method varied greatly. For example, when the disinfectant was $HgCl_2$ it ranged from 5.6% to 67%. At the highest density of about 3000 cm^{-2}, germination rate was around 20%. The germination rate in the last plate was 5.6% as there were only 287 spores in the whole plate. Only plates from the packet method were contaminated (Table 1).

With increasing spore weight, different methods had different problems. For the packet method, when the spores were more than 0.5 g, removing air

TABLE 1. Effects of different sterilization methods and disinfectants on spore germination of *A. reniforme* var. *sinense*.

Disinfectant	Sterilization method	Sterilization time (min)	Spore number/drop	Contamination (%)	Germination (%)±S.D.	Spore color
NaClO	PM	5–6		36.7	40.1 ± 22.9	bleached
NaClO	FM	5–6	235 ± 7.8	0	60.8 ± 8.4	bleached
NaClO	CM	5–6	69 ± 4.6	0	27.4 ± 5.1	bleached
$HgCl_2$	PM	2–3		23.3	39.9 ± 26.3	unchanged
$HgCl_2$	FM	2–3	248 ± 5.7	0	62.6 ± 9.8	unchanged
$HgCl_2$	CM	2–3	75 ± 5.5	0	23.2 ± 3.2	unchanged

Data of spore number/drop were taken after the sterilization; data of contamination and germination were taken after 40 days. PM: packet method; FM: filter method; CM: centrifuge method.

bubbles from the packet became very difficult, and some spores could not be wetted and sterilized. For the centrifugation method, when the spores were 0.5 g, 1.5–2 ml centrifuge tubes were proper; when the spores were 1.5–3 g, 1.5–2 ml centrifuge tubes were too small; 5–7 ml centrifuge tubes and a bigger centrifuge were needed. For the filter method, the spores, regardless of density, could be completely sterilized without any modification of the methodology.

DISCUSSION

When sterilizing spores via the packet method, the spores were kept in the packet during the sterilizing process. However, if the bubbles were not removed completely, the spores did not all come into contact with disinfectant and this caused an increased contamination rate. In addition, the sowing methodology caused the spores to be dispersed unevenly on the culture medium; some plates had spores that were clumped together and some plates had few spores that were spread very far apart. As a result of these discrepancies, the germination rates varied greatly, and confirmed the findings of Ashcroft and Sheffile (2000) that spore germination rate of ferns is inhibited at both high and low densities; proper spore density is important to fern culture.

When sterilizing spores via the centrifuge method, the spores tended to run off when pouring out the used disinfectant solution and used sterilized distilled water. Therefore, after the whole sterilization process, few spores remained, although the spores could be sown in an even density. The results of testing sterilization capacity with this method show that when the spore weight exceeded 1.5 g, larger centrifuge tubes and a larger centrifuge were needed.

When sterilizing spores via the filter method, the spores were kept in the filter funnel during the whole sterilizing process. Thus, spore loss was minimal. Besides this, the sowing spore density could be adjusted evenly through adjusting the volume of the sterilized water used to rinse off the spores from the filter paper.

Given these observations, we conclude that the filter method is an effective way to sterilize spores. It is not only simple and convenient, but can be used to

sterilize many spores at one time and it minimizes spore loss. It also allows spores to be sown in an even density.

The results of this study showed that both $HgCl_2$ and NaClO were effective disinfectants. Since NaClO bleached the spores, it was difficult to judge whether the spores were rinsed off from the centrifuge tubes and filter papers thoroughly or not. However, $HgCl_2$ is not only extremely toxic to spores but also to the environment. Thus, for normal *in vitro* culture, it is better to use NaClO. $HgCl_2$ might be used in cases where the spores are difficult to sterilize or become too bleached. Because of the toxicity of $HgCl_2$, it should be handled very carefully, and the waste of $HgCl_2$ should be detoxified or sent to a waste disposal department for detoxification.

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QUARTERLY JOURNAL OF THE AMERICAN FERN SOCIETY

Obituary: Alice Faber Tryon (1920–2009)
Gerald J. Gastony, David S. Barrington, and David S. Conant **231**

Obituary: Prem Kumar Khare (1946–2009)
Rama Shankar and R. C. Srivastava **236**

***Isoëtes todaroana* (Isoëtaceae, Lycopodiophyta), a New Species from Sicily (Italy)**
Angelo Troia and Francesco M. Raimondo **238**

On *Neolepisorus emeiensis* and *N. dengii* (Polypodiaceae) from China
Xiao-si Guo and Bin Li **244**

***Botrychium ascendens* W. H. Wagner (Ophioglossaceae) in Newfoundland and Notes on its Origin**
Peter F. Zika and Donald R. Farrar **249**

Spore Maturation and Release of Two Evergreen Macaronesian Ferns, *Culcita macrocarpa* and *Woodwardia radicans*, along an Altitudinal Gradient
María L. Arosa, Luis G. Quintanilla, Jaime A. Ramos, Ricardo Ceia, and Hugo Sampaio **260**

Nutrient Levels Do Not Affect Male Gametophyte Induction by Antheridiogen in *Ceratopteris richardii*
Asya Ayrapetov and Michael T. Ganger **273**

Transplanting Tree Ferns to Promote Their Conservation in Mexico
Ana Alice Eleutério and Diego Pérez-Salicrup **279**

Mycorrhizal Associations in Ferns from Southern Ecuador
Marcus Lehnert, Ingrid Kottke, Sabrina Setaro, Linda F. Pazmiño, Juan Pablo Suárez, and Michael Kessler **292**

Differences In Post-Emergence Growth Of Three Fern Species Could Help Explain Their Varying Local Abundance
Kai Rünk and Martin Zobel **307**

The Function of Trichomes of an Amphibious Fern, *Marsilea quadrifolia*
Tai-Chung Wu and Wen-Yuan Kao **323**

SHORTER NOTES

***Isoetes duriei* New to Lebanon**
Lytton J. Musselman and Mohammad S. Al-Zein **333**

Erratum **335**

Referees for 2009 **336**

Table of Contents for Volume 99 **337**

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Obituary: Alice Faber Tryon (1920–2009)

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Alice Faber Tryon became a member of the American Fern Society in 1946 and in 1978 was elected to honorary membership, a special category of membership for those who have made outstanding contributions to the study of ferns. An eminent student of ferns and their spore morphology, she was born Alice Elizabeth Faber in Milwaukee, Wisconsin on August 2, 1920 (according to her sister Jane, she celebrated her birthday on August 1, although her birth certificate reads August 2). She was the second of three children of Arthur H. and Laura Bindrich Faber, and all four of her grandparents had roots in Germany. Known in her family as an ambitious and hardworking woman, Alice was *Aunt Fern* to her nieces and nephews. Alice graduated from the Milwaukee State Teacher's College, now the University of Wisconsin at Milwaukee, in 1941. After several years teaching in public schools, she went to the University of Wisconsin at Madison where she met Rolla M. Tryon Jr. and married him on March 16, 1945. This initiated a happy and enduring domestic

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Photograph by Gerald Gastony at Alice Tryon's Azalea Trace apartment in 2005.

partnership and a research synergism whose productivity has nourished pteridologists throughout the world. Also in 1945, she completed her master's thesis at Wisconsin, began her doctoral studies there under Rolla's direction, and moved with him to the University of Minnesota where Rolla served briefly as an Assistant Professor. The couple moved to the Missouri Botanical Garden in St. Louis in 1947 where Alice completed her doctoral degree at Washington University in 1952.

Alice's life work was the study of fern diversity. During her career, she published nearly 50 contributions to the literature on ferns, including three full-length books. Spores have always been prominent in Alice's work, beginning with her master's thesis, which addressed the taxonomic utility of spore characters in the spikemoss genus *Selaginella*. Her doctoral dissertation analyzed the diversity and taxonomy of the New World species of *Pellaea*, a genus of xerically adapted ferns of the Pteridaceae, a family that remained central to her work during the first half of her career. Her time in St. Louis was followed by a year at the University of California at Berkeley where Rolla was a Research Associate during 1957. In 1958, she and Rolla joined the staff of the Gray Herbarium at Harvard University, where her next major focus was a monograph (1962) of the Andean alpine gymnogrammoid genus *Jamesonia*. Following this, she monographed the closely related Andean-centered gymnogrammoid genus *Eriosorus* (1970). To these revisions, she added papers on reproductive biology and biogeography of the Pteridaceae, notably studies of apogamy in *Pellaea* (1968, 1972), and of incipient heterospory in *Platyzoma* (1964, 1967).

Fern spores were the central focus of Alice's interests in the second half of her career, resuming an interest in spores first expressed in her master's research on the spores of *Selaginella* (1945, 1949). At Harvard, she played a central role in introducing the scanning electron microscope as a research and teaching tool, pioneering its use in the study of fern spores. Prominent among her contributions on spores are her works on evolutionary and ecological trends in spore features (1964, 1973, 1986, 1990), including her study of the specialized spore surfaces of the myrmecophytic ferns (1985). Her book with Bernard Lugardon, *Spores of the Pteridophyta* (1991), is likely to remain the authoritative reference on spore morphology for decades to come.

Alice's professional and personal history is inextricably tied to that of her husband, Rolla M. Tryon, Jr. (1916–2001). Their jointly published work most notably includes *Ferns and Allied Plants with Special Reference to Tropical America* (1982), an in-depth survey of fern diversity with emphasis on the New World tropics. This monumental book, containing numerous photographs by Walter H. Hodge, continues to provide many taxonomic hypotheses that are testable by today's molecular techniques. Together, Alice and Rolla mentored a group of graduate students who have gone on to be prominent in pteridology (see discussion in Gastony *et al.*, 2002). They organized and taught their *Fern Biology in Mexico* course with Ramón Riba, one of their students, five times between 1971 and 1981. This stimulating opportunity to do science with ferns in the field was a formative experience for all participating students.

Alice and Rolla had a lifelong investment in creating venues in which scientists could interact in the kinds of informal, relaxed settings that lead to the development of new insights about the botanical world, especially the ferns, but in much broader contexts as well. Prominent among these is the Missouri Botanical Garden's annual *Systematics Symposium*, initiated by Rolla and Alice during their time in St. Louis, and the *New England Fern Conference*, which Rolla and Alice inaugurated in 1970.

Following her arrival in New England in 1958, Alice was deeply involved in the New England Botanical Club. She was elected its first woman member in 1968. After serving as recording secretary and vice president, Alice was elected the club's first woman president in 1978. During her time as president, the club inaugurated several successful programs, including a focus on New England's rare and endangered species in the 1979 symposium *Rare and Endangered Plant Species in New England*, the proceedings of which were published in 1980. Her interest in New England and long-time residence there led Alice to develop her last book, *The Ferns and Allied Plants of New England* (1997), coauthored with Robbin Moran. This book is notable for its images of the plants, including both the classic photographs of Robert L. Coffin and the more recent work of noted botanist and photographer Walter H. Hodge. For this book, Alice included spore images for each of the New England pteridophytes, a fortunate inclusion for students of New England Pleistocene biogeography who find this resource invaluable in their analyses of palynological cores.

After their retirements from Harvard, Alice and Rolla retired to Florida in 1989. While there, they continued their pattern of supporting small venues for the discussion of scientific ideas by founding the Institute for Systematic Botany and the Tryon Lecture Series at the University of South Florida in Tampa. In 1990, Alice and Rolla were honored with a festschrift occupying pages 222–339 of *Annals of the Missouri Botanical Garden* volume 77. This tribute to the Tryons featured an opening photograph of them at the portrait of Daniel C. Eaton (first American pteridologist) at Harvard University, an introductory summation of their contributions to pteridology, a closing photograph of them by Walter H. Hodge, and contributed papers by the following: Cathy A. Paris and David S. Barrington; R. James Hickey; Robbin C. Moran; Alan R. Smith; Robert G. Stolze; Gillian A. Cooper-Driver; Ramón Riba and Irma Reyes J.; David S. Conant; David S. Barrington; Gerald J. Gastony; Christopher H. Haufler, Michael D. Windham, and Thomas A. Ranker; Karl U. Kramer; and Diana B. Stein and David S. Barrington.

For more than a decade, Alice's and Rolla's partnership in scholarly work and community outreach about the ferns of Florida were centered at the University of South Florida, ending with Rolla's death in 2001. Following that, Alice moved to the Azalea Trace retirement community in Pensacola, Florida where the Tryons' good friends Walter and Barbara Hodge were already in residence. Among Alice's final acts of scientific altruism were her generous establishments of endowments for the Field Museum in Chicago, the New England Botanical Club, the Alice and Rolla Tryon Pteridophyte Library at the

Pringle Herbarium of the University of Vermont, and the Rolla and Alice Tryon Scholarship Fund in support of the Woman in Science program of the Department of Botany at the University of Wisconsin, Madison.

Comforted by her care-giving friends, Alice died peacefully in her garden apartment at Azalea Trace on March 29, 2009, surrounded by many mementoes of a life happily shared with Rolla and dedicated to advancing our knowledge of ferns. On September 27, 2009, the authors and their wives united Alice's ashes with Rolla's on a ferny hill in northern Vermont. A simple bronze plaque affixed to a boulder at the site records their passing with the following words.

In Memory Of

Rolla M. Tryon Jr. (1916–2001)

And Alice F. Tryon (1920–2009)

Eminent Pteridologists

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Obituary: Prem Kumar Khare (1946–2009)

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Regional Research Institute (Ayurveda) and BSI Itanagar

Born on 1st June, 1946 at Varanasi, Professor P. K. Khare, after completing his Master Degree from Gorakhpur University, Gorakhpur, started his research career from Allahabad University, Allahabad under esteemed guidance of late Professor Divya Darshan Pant, the then Head of Botany Department on various morphological and paleobotanical aspects of pteridophytes. During his research career he discovered *Damudopteris polymorpha* species from Raniganj hills. At the same time he has also discovered stomatal ontogeny of *Psilotum*, *Tmesepteris* species, *Dipteris* (*wallichii*), a rare fern. Simultaneously, he started his professional career as Lecturer in the same department in February, 1974 and continued as Reader as well as Professor of Botany. During 2007–08 he was honored with the prestigious chair of Head of the Botany Department of Allahabad University, Allahabad.

During his professional career Professor Khare guided many research scholars. During his research career he made comprehensive studies on petiolar structure, phytochemical studies of economically and taxonomically important ferns as well as ecology of Pteridophytes of Western Himalaya and Central India. While studying petiolar structure he had given guidance for

establishing petiolar characters as good tool for taxonomic identification of ferns, particularly *Adiantum*, *Asplenium*, *Ophioglossum* and *Pteris* species besides other fern species. For phytochemical studies he studied ferns from Western Himalayas and Central India. To his credit Professor Khare published over 50 research papers in *Journal of Royal Society*, *Annals of Botany*, *Canadian Journal of Botany*, *American Fern Journal*, *International Journal of Pharmacognosy* and other national journals like *National Academy of Sciences*, *Indian Fern Journal*, etc. During the year 2008 Professor Khare was graced with the deliberation of Dr. G. Panigrahi Memorial lecture at the occasion of the Indian Botanical Conference held at Allahabad. Professor Khare left this world on 3rd May, 2009 with his sweet memory to science. He is survived by his wife, one married daughter and one son who recently joined Provincial Civil Service (PPS).

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Isoëtes todaroana (Isoëtaceae, Lycopodiophyta), a New Species from Sicily (Italy)

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ABSTRACT.—*Isoëtes todaroana*, a new species from western Sicily (Italy), is described. Morphological, anatomical and ecological characters are given. The main differential characters are the presence of only two leaf air chambers, rather than four as in all other known species of the genus, and the shape of the scales, which have two lateral rounded lobes and one central spine-like lobe, together with its peculiar calcophilic habitat. So far, the species is known from a single locality.

KEY WORDS.—Lycopodiophyta, Isoëtaceae, *Isoëtes*, Mediterranean area, Italy, Sicily

Four species of *Isoëtes* have been previously reported from Sicily (Troia, 2005): *Isoëtes histrix* Bory, *I. sicula* Tod. [= *I. subinermis* (Bory) Cesca & Peruzzi, =? *I. gymnocarpa* (Gennari) A. Braun], *I. velata* A. Braun, and *I. duriei* Bory. All of these grow on seasonally wet, siliceous soils, except for *I. velata* which colonizes temporary ponds.

Over the last ten years, field, herbarium, and laboratory studies have been conducted by one of us on the genus *Isoëtes* (e.g., Romeo *et al.*, 2000; Troia and Bellini, 2001; Troia, 2005). As part of these studies, a population of *Isoëtes* was located in a wetland near Mazara del Vallo, Western Sicily. Closer inspection of the specimens showed that they differed in several aspects from the other species occurring on the island. Analyses of living and dried plants confirmed that this population represents a unique and previously undescribed species of *Isoëtes*, which is here named and described. For description and nomenclature of megaspores and microspores we followed Hickey (1986) and Musselman (2003), respectively.

Isoëtes todaroana Troia & Raimondo, ***sp. nov.*** TYPE.—ITALY. Sicily: contrada "Critazzo" near Mazara del Vallo, 37°41′07″N, 12°37′05″E, *ca.* 60 m a.s.l., 10 Apr. 2009, *Angelo Troia* (holotype: PAL; isotypes: PAL, FI). **Figs. 1–10**.

Herba perennis amphibia, emergens aut submersa. Cormus trilobus, radicibus dichotomis. Folia 15–30 (−40), erecta vel patentia, 3–6 (−14) cm longa, inferne brevi tractu anguste alato-membranacea, basi usque 3–4 mm, in medio circa 1 mm lata. Duae magnae lacunae et 1–3 fasciculi fibrarum periphaerici (collenchymatosi) in trasversali sectione. Stomata elliptica, 37–70 μm longa, 24–32 μm lata, in facie abaxiali tantum obvia. Velum sporangium

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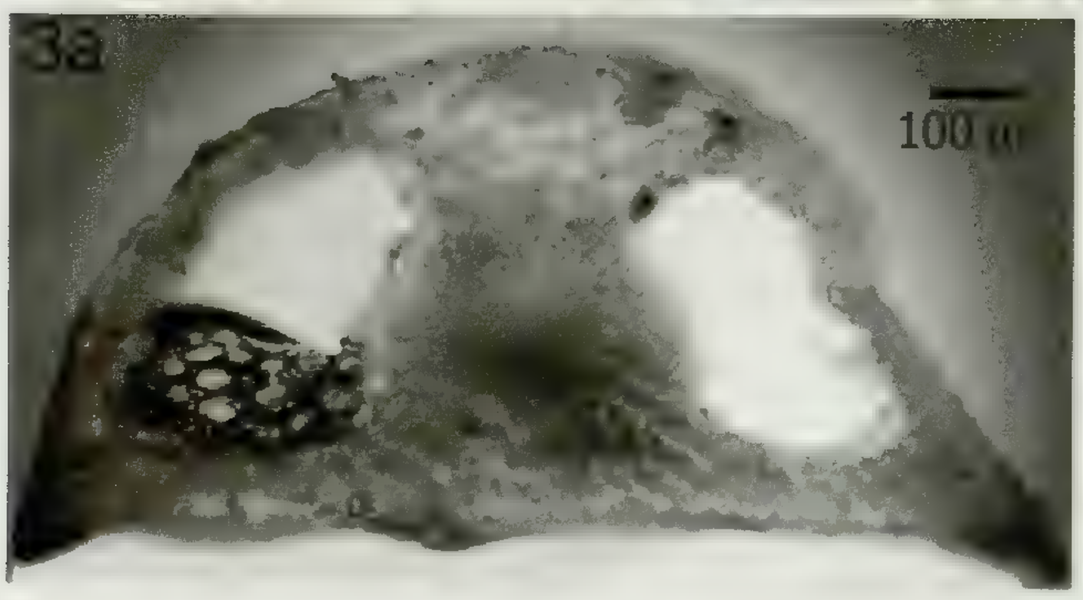
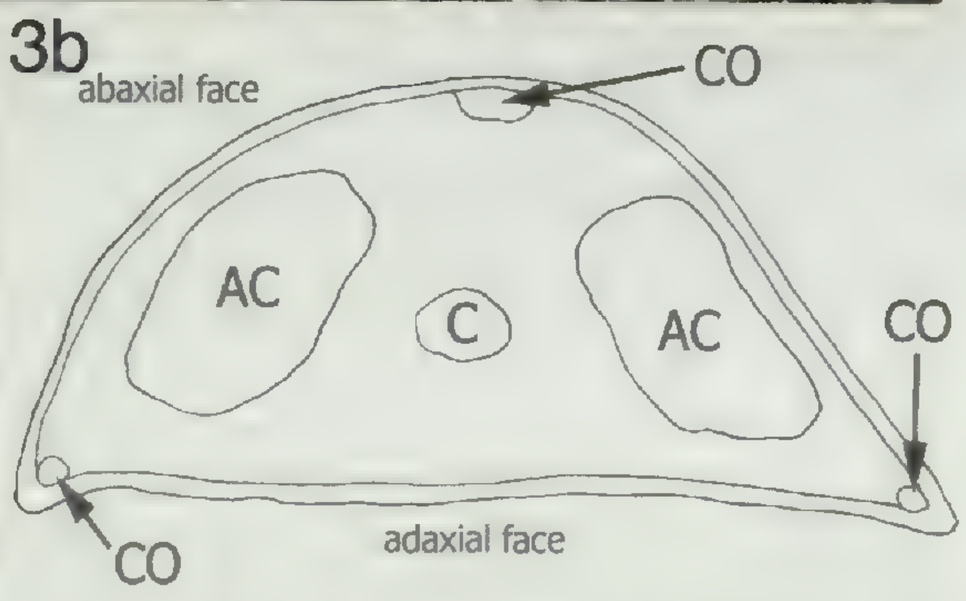
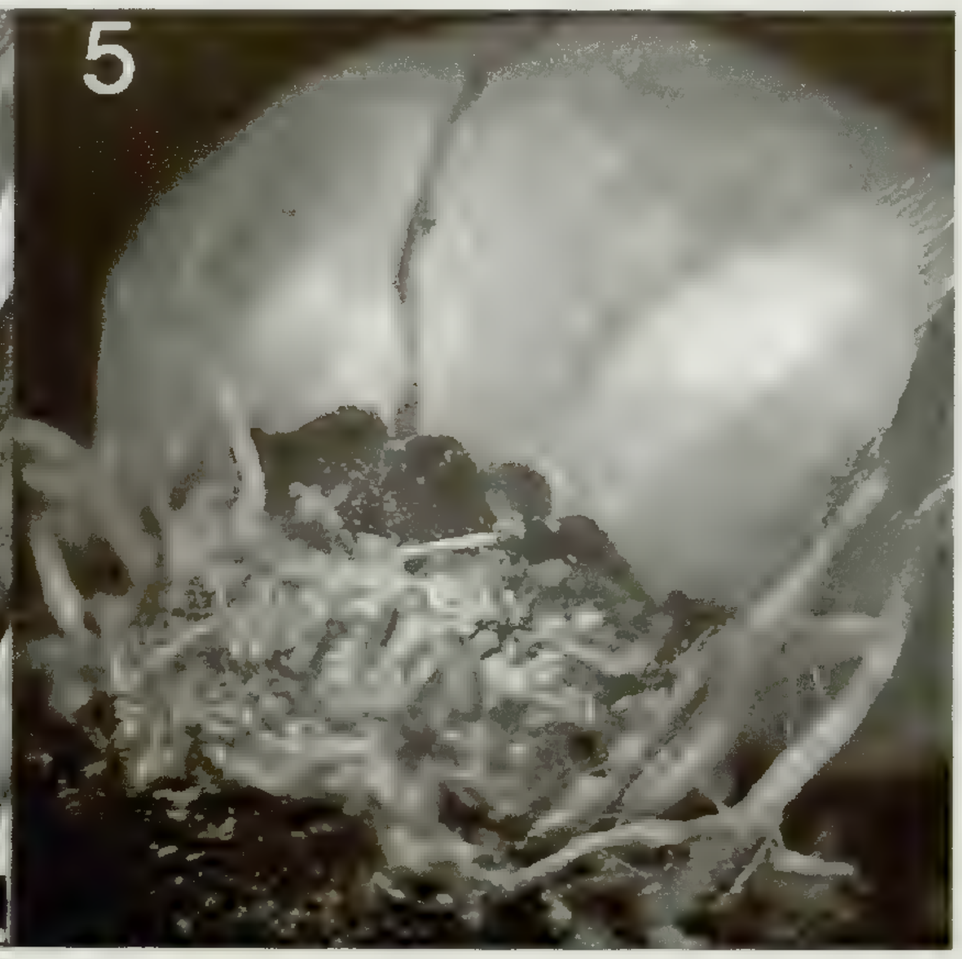


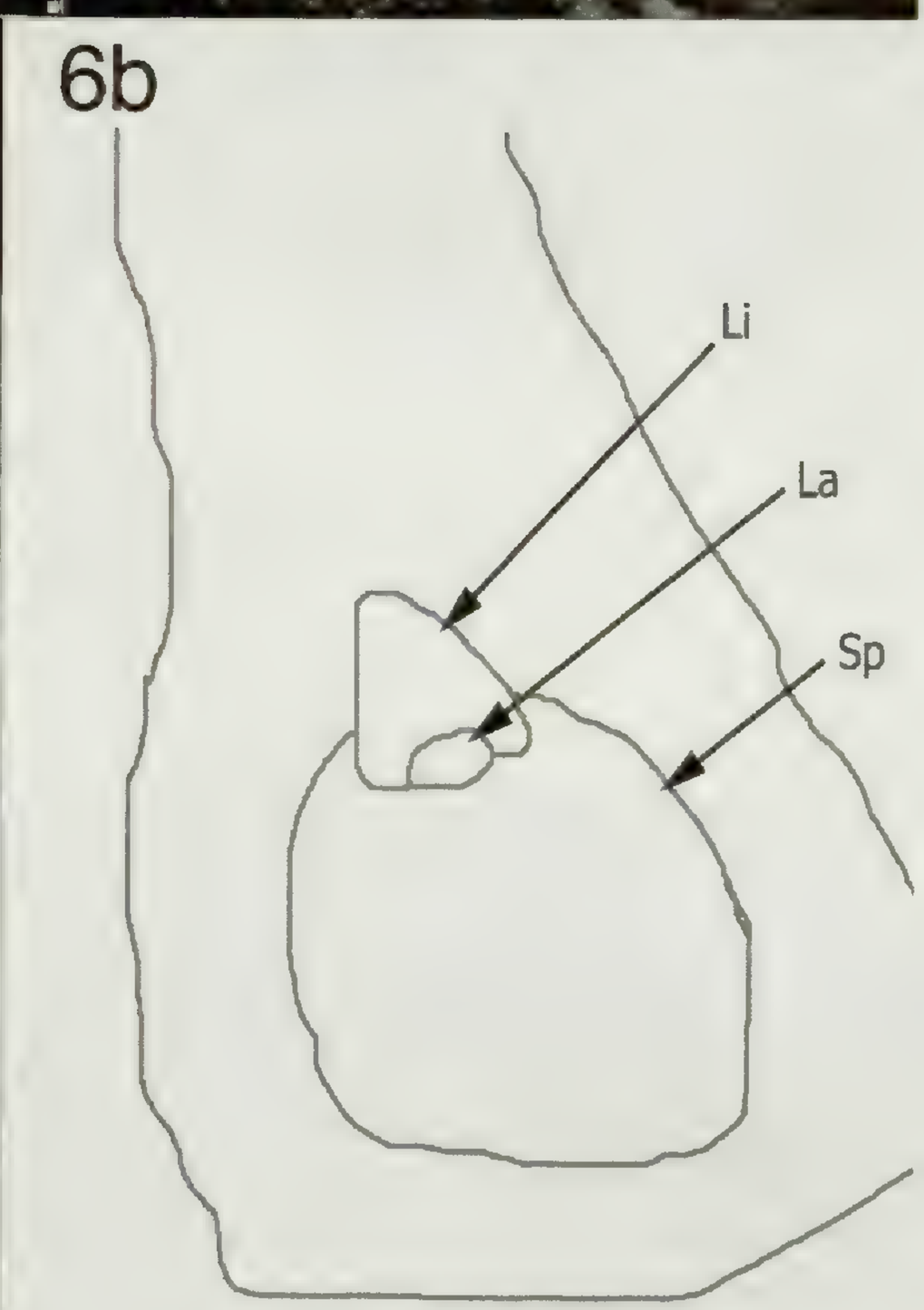


FIGS. 1–3. Morphological and anatomical traits of *I. todaroana*. 1–2. Plants in their habitat (the coin is *ca.* 18 mm in diameter). 3 (a/b). Transverse section of the leaf: AC = air chambers (the one on the left partially covered by the translacunar diaphragm); CO = collenchymatous strands; C = vascular bundle (with at least one intrastelar canal).

obtegens. Ligula membranacea, lanceolata, basi auriculata. Labium breve. Squamae paucae, coriaceae, nigrescentes, lobis lateralibus rotundatis et dente medio aciculari. Macrosporae candidae, sphaeroideae, 420–460 µm, annulo horizontali cinctae, hemispherio superiore tricostatae, undique tuberculatae. Microsporae ovatae, *ca.* 25 µm longae, aculeatae. Habitat in humidis hyeme inundatis.

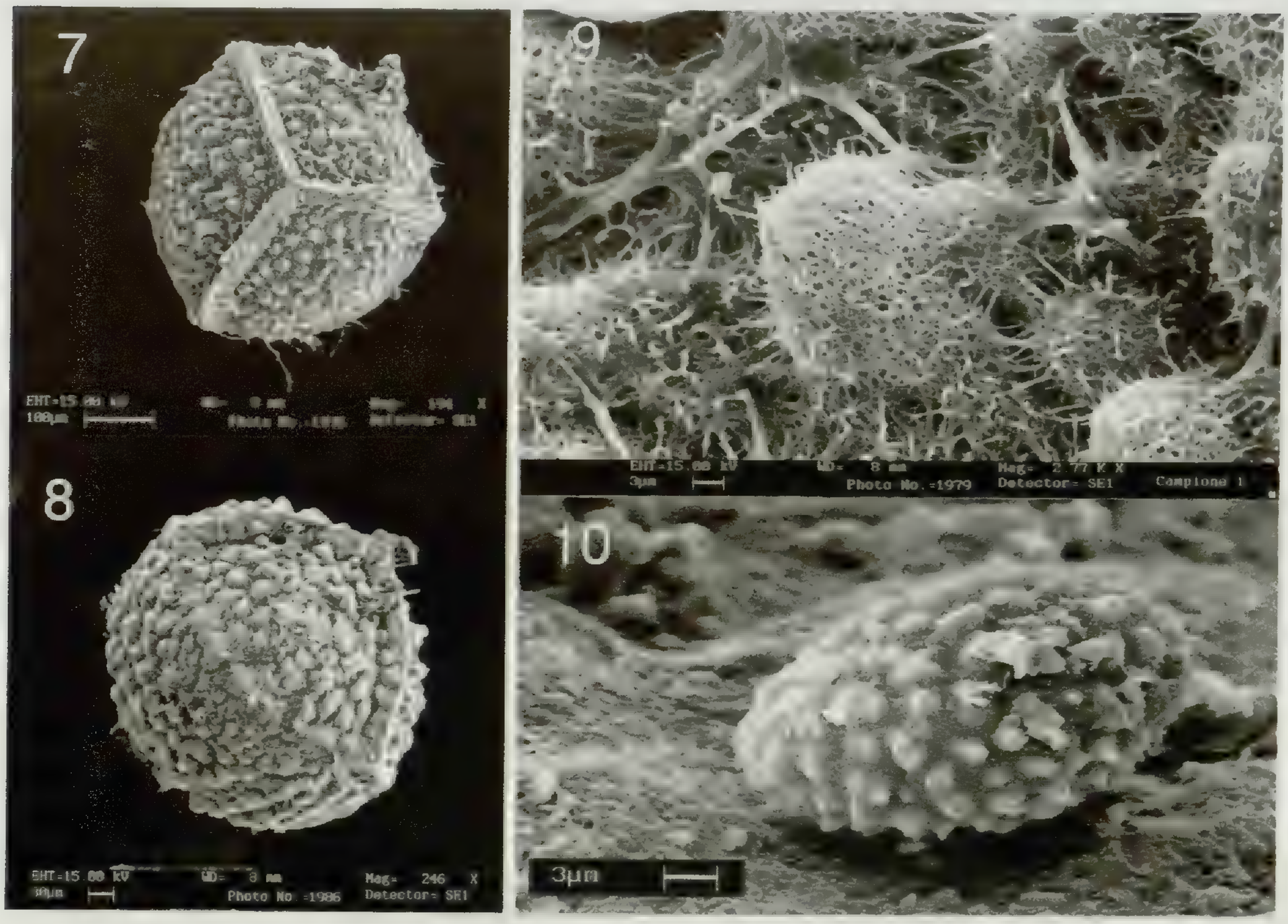
Plants amphibious, emergent or submerged in temporary ponds, losing their leaves in the dry summer season. Stem (corm) trilobate, with dichotomous roots. Leaves 15–30(−40), patent to erect, narrowly lanceolate, 3–6 (−14) cm long, 3–4 mm wide at base, *ca.* 1 mm wide at mid-length. Alae proximally hyaline or transluscent, *ca.*1 mm wide at the sporangium, gradually narrowing distally. Subula semiterete, adaxially flat, abaxially convex. Leaf epidermis with cuticular ornamentation, "cuticular pegs" (sensu Prada and Rolleri, 2005, = "cuñas cuticulares" sensu Rolleri and Prada, 2007) well developed, evident as continuous longitudinal ridges. Stomatal complexes in rows, elliptic, 37–70 µm long, 24–32 µm wide, confined to the abaxial surface. Hypodermal collenchymatous bands one to three, the two marginal bands sometimes absent. Air chambers two, with translacunar diaphragms. Velum complete. Ligule *ca.* 1 mm long, membranaceous, broadly lanceolate, auriculate at base.





FIGS. 4–6. Morphological and anatomical traits of *I. todaroana*. 4. Surface view of the leaf epidermal cells. Note the cuticular ornamentation, the cuticular pegs well developed, evident as continuous longitudinal ridges, and the epiphytic diatoms. 5. Scales. 6 (a/b). Adaxial face of the leaf base: Li = ligula; La = labium; Sp = (mega)sporangium.

Labium shorter than ligule. Scales few, black, with two lateral rounded lobes and one (usually short) central spine-like lobe. Megaspores 420–460 µm in diameter, white, subtriangular in polar view, tuberculate. Microspores *ca.* 25 µm long, aculeate.



FIGS. 7–10. SEM images of *I. todaroana* megaspores and microspores. 7. Proximal view of megaspore. 8. Equatorial view of megaspore. 9. Megaspore (detail of a single tubercle). 10. Distal view of microspore.

ETYMOLOGY.—This new species is dedicated to the Sicilian botanist Agostino Todaro (1818–1892), in recognition of his contribution to the pteridological flora of Sicily.

ECOLOGY.—The type locality is a temporary wetland that dries out during the summer. It is a remnant of a wider wetland that has been "reclaimed" and converted to farming land that surrounds and encloses the type locality. The natural vegetation, although altered, is well represented with a mosaic of communities, with species such as *Bolboschoenus maritimus* (L.) Palla, *Eleocharis palustris* (L.) Roem. & Schult., *Scirpus cernuus* Vahl, *Mentha pulegium* L., *Oenanthe* sp., *Lythrum* sp., *Tamarix* sp., *Romulea* sp., etc. The wetland hosts the last remnants of a peculiar freshwater invertebrate fauna; a preliminary investigation has led, for example, to the discovery of a small population of the notostracan *Lepidurus apus lubbocki* (Crustacea), a "living fossil" that was considered extinct in Sicily (F. Marrone, pers. comm.).

The wetland exists on a peculiar geological substrate of calcareous sandstones with a thin layer of clays on top (hence the local name, "Critazzo", which in Sicilian dialect refers to clays). The soil pH around the *Isoëtes* (determined electrometrically with two replicates from 20 g of soil and measured in distilled water with a dilution ratio of 1: 2.5) was found to be

basic, 8.5–8.9. As far as we know, the other species occurring in Sicily consistently grow on acid soils, so that this site, although geologically complex, is unusual, a fact that deserves further investigations.

DISTRIBUTION.—*Isoëtes todaroana* is only known so far from the *locus classicus*, in an area of about 200 × 100 m. It is possible that other populations may be found in the future, in Sicily and elsewhere in the Mediterranean area.

CONSERVATION STATUS.—The single known population is threatened by farming and other human activities (waste dumping, land reclamation, summer fires, etc.). On the basis of the current "IUCN Red List Categories and Criteria" (IUCN, 2001), the species is rated "Critically Endangered" [CR B1ab(iii)].

Urgent measures are needed to protect the site; considering that it is practically unexplored, it is possible that other rare or endangered species are present, in addition to *Isoëtes todaroana* and *Lepidurus apus lubbocki* already mentioned. However, it is clear that it is a strategic area for migrating birds and it hosts communities that can be referred to the habitat "Mediterranean temporary pond", considered a "priority" habitat by the Council of the European Communities 92/43/EEC Directive. Our proposal is to include the site, currently not protected, inside the adjacent "Site of Community Interest" code ITA010014, established to protect habitat and species according to the mentioned Directive. Since this inclusion will not be made in a short time and will not automatically guarantee the protection of species and habitats, we also suggest considering other actions (e.g., agreements with the owners, territory planning restrictions, land purchase) as soon as possible.

TAXONOMIC OBSERVATIONS.—The shape of the scales, and particularly the presence of only two leaf air chambers rather than four as in all other species of the genus (Rolleri and Prada, 2007), are the main differentiating characters of this species. Its systematic relationships are difficult to assess merely on the basis of anatomy and morphology, owing to parallel and convergent morphological evolution in the genus (Hickey, 1986; Hoot *et al.*, 2006; Bolin *et al.*, 2008). The presence of scales, sometimes transitioning into phyllopodia, and collenchymatous strands suggests a link between this species and the Mediterranean "terrestrial" taxa, and the megaspore ornamentation, in particular, vaguely suggests a connection with *I. histrix* and *I. sicula*. However, as shown by Bolin *et al.* (2008), the latter two species, although morphologically similar, are not immediately related. On the other hand, the fibrillose megaspore surface background (Fig. 9) suggests a relationship with other species, e.g., the amphibious *Isoëtes velata*.

Further studies are in progress to add to the knowledge of the new species and shed light on its relationships.

ACKNOWLEDGMENTS

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Cecilia Macluf for their valuable comments. Financial support from Regione Siciliana (L.R. 25/93) and Università degli Studi di Palermo (Fondi di Ateneo, ex 60%, titolare F.M. Raimondo) is gratefully acknowledged.

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On *Neolepisorus emeiensis* and *N. dengii* (Polypodiaceae) from China

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Abstract.—*Neolepisorus dengii*, *N. dengii* f. *hastatus* and *N. emeiensis* f. *dissectus* should be considered synonyms of *N. emeiensis*. This treatment is justified on the basis of complete intergradation of the frond forms that supposedly separate these taxa. The intergradation can be found on the same individual.

Key Words.—*Neolepisorus*, China, synonyms

Neolepisorus occurs in tropical and subtropical Asia and Africa. It has one center of distribution in the Yangtze River area and south and southwest China. Its species are endemic to mainland China except for one endemic to Madagascar, one in Indo-Himalayas, upper Burma, northern Thailand, Indo-China and China, and a third in Japan, Philippines and China (including Taiwan). As originally proposed by Ching (1940), *Neolepisorus* consisted of three species: *N. lastii* (Baker) Ching, *N. ovatus* (Bedd.) Ching, and *N. ensatus* (Thunb.) Ching. Subsequently Ching and Shing (1983) published regional taxonomic revisions of Chinese *Neolepisorus* and recognized 10 species, a conclusion with confirmed by Lin Youxing (2000).

Based on the study of specimens at KUN, PE, SZ and WUK, we came to doubt the establishment of a species and two forms that were named by Ching and Shing (1983). They claimed that *Neolepisorus emeiensis* and *N. dengii* differed in the shape of the lamina base. Furthermore, the published two forms, *N. emeiensis* f. *dissectus* and N. *dengii* f. *hastatus*, also based on differences in the shape of the lamina base. Our field observations and identification of *Neolepisorus* from southern Shaanxi Province, especially the Dabashan Mountain region, have confirmed these forms intergrade and therefore do not merit taxonomic recognition. We suggest that *N. dengii*, *N. dengii* f. *hastatus* and *N. emeiensis* f. *dissectus* be considered synonyms of *N. emeiensis*.

Neolepisorus emeiensis Ching et K. H. Shing, Acta Phytotax. Sin. 21(3): 271, f. 2:1, 1983. TYPE.—China. Sichuan: Mount Emei, Hongchunping, 1150 m [erroneously as "1500" m in the protologue], 27 August 1964, *K. J. Kuan et al.1836* (Holotype, PE!)

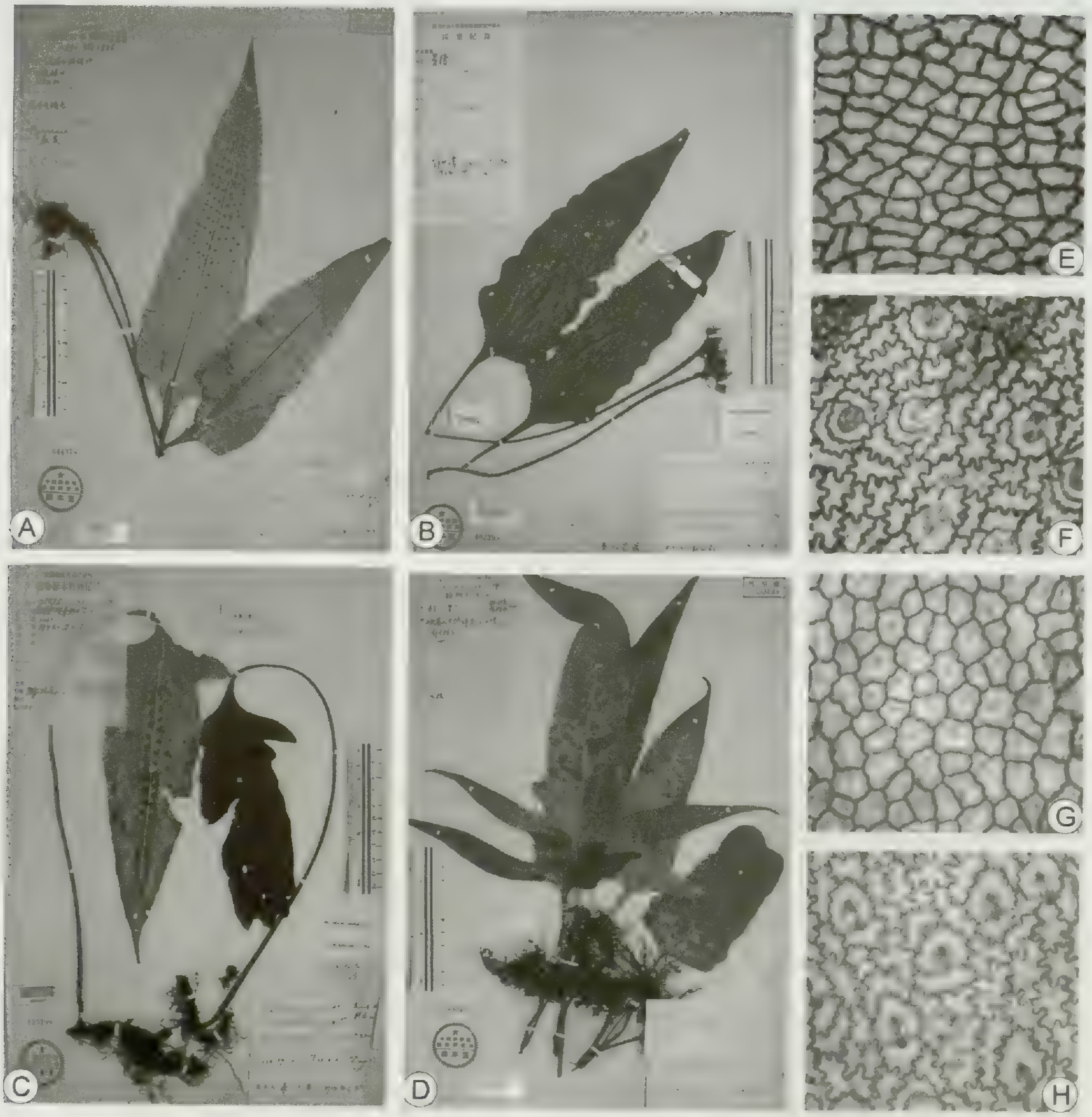

FIG. 1. Herbarium specimens and epidermal structures of two species of *Neolepisorus*. A. The holotype of *Neolepisorus emeiensis* (*K. J. Guan & W. C. Wang 1836*, PE). B. The holotype of *N. dengii* (*S. W. Deng 90002*, PE). C. The holotype of *N. dengii* f. *hastatus* (*P. S. Wang 75473*, PE). D. The holotype of *N. emeiensis* f. *dissectus* (*G. X. Shing & K. Y. Lang 1143*, PE). E. Upper epidermis of *N. emeiensis* (*T. L. Dai* 107331, WUK). F. Lower epidermis of *N. emeiensis* (*T. L. Dai* 107331, WUK). G. Upper epidermis of *N. dengii* (*T. P. Wang 8542*, WUK). H. Lower epidermis of *N. dengii* (*T. P. Wang 8542*, WUK).

Neolepisorus dengii Ching et P. S. Wang, Acta Phytotax. Sin. 21(3): 272, f. 2:3, 1983, syn. nov. TYPE.—China. Guizhou: Guiyang, 12 April 1936, *S. W. Deng 90002 bis* (Holotype, PE!)

Neolepisorus dengii Ching et P. S. Wang f. *hastatus* Ching et P. S. Wang, Acta Phytotax. Sin. 21(3): 271, f. 2:4, 1983; TYPE.—China. Guizhou: Anshun, 1260 m, 17 December 1977, *P. S. Wang 75473* (Holotype, PE!)

TABLE 1. Comparison of *Neolepisorus emeiensis* and *N. dengii*.

Character	*N. emeiensis*	*N. dengii*
Habitat	wet forest floors	shady places or on rocks
Altitude	500–1800 m	500–1300 m
Rhizomes	long-creeping	long-creeping
Scales	brown, ovate-lanceolate	dark brown, lanceolate
Frond texture	lightly coriaceus at dry	thickly chartaceous at dry
Frond length	24–28 cm	20–27 cm
Frond shape	broadly oblong-lanceolate, 6–7 cm wide, widest at base	triangular-lanceolate, 5–8 cm wide, widest at base
Frond base	obliquely cuneate	lightly hastate or obliquely cuneate
Frond color	yellow-green at dry	brown or brown-green at dry
Lateral veins	mostly flat, visible, free	lightly oblique, visible
Sori	round, larger, borne in 1–2 rows between the lateral main veins	round, smaller, borne in 1–2 row between the lateral main veins

Neolepisorus emeiensis Ching et K. H. Shing f. *dissectus* Ching et Shing, Acta Phytotax. Sin. 21(3): 271, f. 2:2, 1983. TYPE.—China. Sichuan: Mount Emei, 1000 m, 1 September 1963, *K. H. Shing et K. Y. Lang 1143* (Holotype, PE!)

DISTRIBUTION AND HABITAT.—China (Sichuan, Hubei, Jiangxi, Guizhou, Southern Shaanxi); forests on hills or slopes or in valleys; 500–1800 m.

The protologue of *Neolepisorus dengii* (Fig. 1B) claims that it differs from *N. emeiensis* (Fig. 1: A) by triangular-lanceolate fronds, slightly hastate lamina bases, thickly chartaceous, brown or brown-green laminae when dry, and lateral veins slightly oblique. Otherwise, the two species do not differ. After examining more material of *N. emeiensis*, we conclude that its frond shape falls within the range of variation of *N. dengii* (Table 1).

To further test for differences, we studied the epidermis of *Neolepisorus dengii* and *N. emeiensis* with an optical microscope. We found that the two species have similarly shaped epidermal cells, including the distribution, structure, and type of stomata (Fig. 1: E, F, G, H). The arrangement of the upper epidermal cells in two species was also the same. There are copolocytic and coaxillocytic stomata in the lower epidermis. Thus we found no differences in epidermal characters between the two species.

Neolepisorus dengii f. *hastatus* (Fig. 1: C) and *N. emeiensis* f. *dissectus* (Fig. 1: D) differ from *N. emeiensis*, respectively, only by the base of fronds hastate or with 1 or 2 pairs of long lanceolate segments. In observing many individuals in southern part of Shaanxi Province, we found that the fronds lobed or with 1–2 pair of segments (from lobate to triangular to lanceolate in shape) at base often occurs in *N. emeiensis* (Fig. 2). Through a wide range of herbarium and field investigations, two foliar variations in *N. emeiensis* have been determined: 1) from broadly oblong-lanceolate, triangular-lanceolate to halberd-shaped in shape, and 2) from obliquely cuneate to hastate at base. These two foliar variations are the basis of the two named forms. Thus we

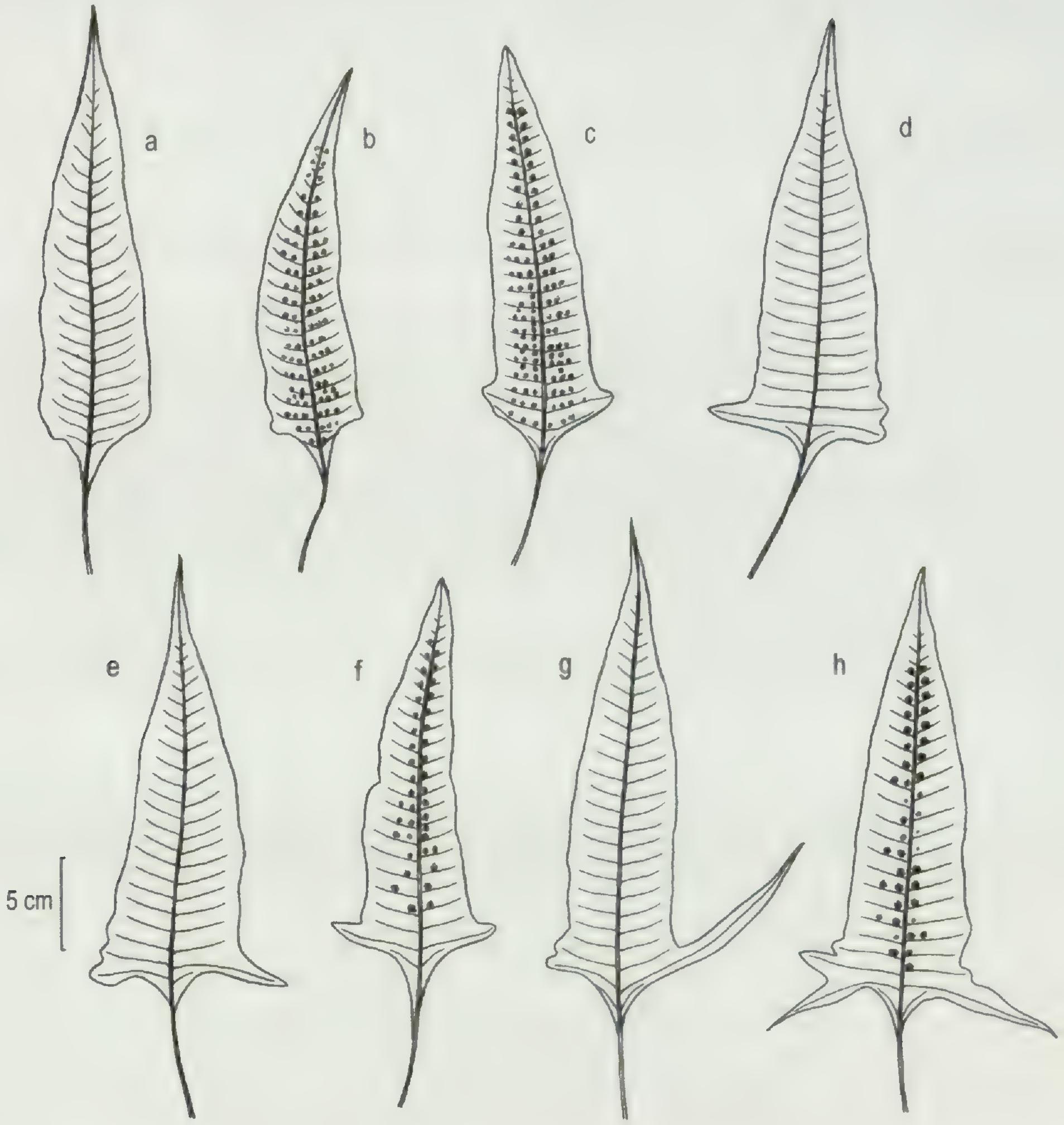

FIG. 2. Intergradation in frond form in a single individual of *Neolepisorus emeiensis* (*Y. S. Chen et al. 2206*, WUK). A, B, Frond broadly oblong-lanceolate, base obliquely cuneate. C–F. Frond lanceolate or triangular-lanceolate, base lightly hastate. G, H. Frond triangular-lanceolate, base with 1 or 2 pairs of long lanceolate segments.

concluded that the shape of the lamina base is an unstable character, which could not be used to establish a new taxon.

All supposedly diagnostic characters of *Neolepisorus dengii*, *N. dengii* f. *hastatus* and *N. emeiensis* f. *dissectus* are variable and fall within the range of that found in *N. emeiensis*. Therefore, *N. dengii*, *N. dengii* f. *hastatus* and *N. emeiensis* f. *dissectus* should be considered synoymyms of *N. emeiensis*.

ACKNOWLEDGMENTS

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Botrychium ascendens W. H. Wagner (Ophioglossaceae) in Newfoundland and Notes on its Origin

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Abstract.—*Botrychium ascendens* is reported from Fogo Island in Newfoundland as an addition to the flora of the province. Fogo Island plants are identical to plants in western North America, including those from the type locality, in comparisons of leaf morphology, spore size, and allozyme expression. Comparisons are made with related and confusing taxa, *B. campestre*, *B. crenulatum*, *B. lineare*, and the American genotype of *B. lunaria*. Newfoundland plants display a high level of fixed heterozygosity and large spore size, indicating they are allotetraploid, and supporting suggestions that *B. ascendens* is derived from ancient hybridization between *B. crenulatum* and the *B. lineare/campestre* complex. The current distribution of *Botrychium ascendens* and its putative parents suggest it probably originated in western North America and migrated across northern Canada to Newfoundland.

Key Words.—*Botrychium ascendens*, leaf morphology, allozymes, taxonomy

Canada's Maritime Provinces of Nova Scotia, New Brunswick, Newfoundland and Québec are reported to harbor 14 species of *Botrychium* (Cody and Britton, 1989; Wagner and Wagner, 1993; Kartesz, 1999), not including *Botrychium ascendens* W. H. Wagner, a species commonly found throughout western North America, east to central Alberta, with an outlier collection from northern Ontario (Wagner and Wagner, 1993).

Wagner and Wagner (1990) tentatively reported a 1985 Britton and Anderson collection from Fogo Island in Newfoundland as *Botrychium campestre* W.H. Wagner & Farrar. The Wagners were uncertain because the pressed material was scanty. They wished to see a larger collection to study the morphological variation in the population. A traditional problem in the elucidation of species in *Botrychium* subgenus *Botrychium* is that much herbarium material consists of only one to a few plants, these often folded or shriveled, and thus difficult to classify. The most useful samples have leaves pressed with all pinnae flat and clearly visible, and have a minimum of 10–20 plants showing the variability within the population. (Underground parts are not diagnostic in moonwort species identification. Carefully harvesting the above-ground leaf by cutting at ground level allows the below-ground bud to produce new leaves in subsequent years.) Limited samples fail to show typical population variation, and if the individual specimens are small, they may resemble juvenile or small plants of other taxa. Diagnostic features observable in the field, such as stature, color, fleshiness and luster, are rarely noted by collectors on museum labels.

To overcome these difficulties, we visited the Fogo Island population to secure an adequate sample.

Wagner and Wagner (1986) reported a chromosome number of n = 90 for B. ascendens, indicating it to be a tetraploid species. Using *rbcL* sequence comparisons Hauk (1995) found *Botrychium ascendens* clustered with the lineage including *B. campestre* and *B. lineare* W. H. Wagner, suggesting this lineage contributed the chloroplast genome to *B. ascendens*. Using allozymes at a smaller number of loci (6) than the current study, Hauk and Haufler (1999) found the non-chloroplast genome of *B. ascendens* clustered with *B. crenulatum* W. H. Wagner, thus suggesting an allopolyploid origin of *B. ascendens* through ancient hybridization between *B. campestre*/*B. lineare* and *B. crenulatum*. In this paper we examine the variation of the Newfoundland population, and compare it with the morphology and allozymes of *B. ascendens* and related species of known provenance from elsewhere in North America, to confirm the identity of the Newfoundland plants and provide further evidence of their origin. We also present morphological characters useful in separating *B. ascendens* from *B. campestre* and *B. lineare*.

Materials and Methods

Plant collection.—Plants of *Botrychium ascendens* were obtained from Sandy Cove on Fogo Island in Newfoundland on June 26, 2001. Plants representing the observed variation were collected by cutting leaves at ground level and storing them in plastic containers in an ice chest until processing. The plants were divided into groups, one for immediate pressing and one maintained fresh for enzyme electrophoresis. After samples for electrophoresis were removed from the common stalk, the latter were also pressed for herbarium vouchers. Vouchers of all plants in the study are deposited in the Ada Hayden Herbarium of Iowa State University (ISC).

Voucher specimens are labeled: CANADA: **Newfoundland:** Fogo Island, Sandy Cove, 7 July 1985, *Britton 10671 & Anderson* (MICH); Fogo Island, Sandy Cove, elev. ca. 3 m, 49° 42.5′ N, 54° 5.0′ W, 26 June 2001, *Zika 16334* (CAN, ISC, MICH, MT, NFM, WTU). The population is quite local and restricted to 80 meters of low sand dunes southwest of Route 334, at Sandy Cove, by a sandy beach near Tilton. About 150 stems were seen on 26 June 2001. The fern's associates included *Carex nigra* (L.) Reichard, *Festuca rubra* L., *Achillea*, *Linnaea*, *Fragaria*, *Taraxacum*, *Aralia nudicaulis* L., *Ranunculus acris* L., *Equisetum arvense* L., and *Botrychium lunaria*.

Allozyme electrophoresis.—From an ongoing study of all moonwort *Botrychium* by Farrar, we constructed genetic profiles obtained through starch-gel enzyme electrophoresis for species relevant to this study. The source of genetically analyzed plants is listed in Table 1. The number of individuals sampled per site ranged from 1 to 92 depending on population size. The average number of plants per site was 10.6.

Single leaves were cut at ground level and kept cool until processing. Approximately one-centimeter segments were removed from the common stalk

TABLE 1. Source of collections used in genetic analysis, including the number of sites sampled and total number of plants analyzed. Details of site locations can be obtained from Farrar.

Botrychium	State or Province	# Sites	# Plants
ascendens	Alaska	6	84
	California	1	12
	Montana	5	19
	Nevada	1	3
	Newfoundland	1	10
	Oregon	2	34
	Washington	3	24
campestre	Alberta	1	3
	Iowa	5	47
	Michigan	1	16
	Minnesota	7	74
	Montana	4	17
	South Dakota	3	38
	Wyoming	1	1
crenulatum	Alberta	1	6
	California	9	41
	Montana	5	19
crenulatum	Nevada	3	15
	Oregon	5	27
	Utah	2	25
lineare	Alaska	1	2
	Colorado	2	12
	Montana	5	34
	Oregon	2	15
	South Dakota	3	11
	Washington	1	1
	Wyoming	3	11
	Yukon	1	1
*lunaria**	Alaska (coastal)	18	476
	Colorado	2	51
	Michigan	3	25
	Ontario	1	15
	Washington	5	16
Total		113	1185

*The common American genotype differs genetically from European *B. lunaria* suggesting it could be described as a new species (Stensvold 2008).

(petiole) and ground with mortar and pestle in a phosphate-polyvinypyrrolidone extraction buffer (Cronn *et al.*, 1997). Grindates were stored at −70°C until used, at which time they were spun at 12,000 rpm for two minutes to produce a clear enzyme-containing supernatant. The extracts were absorbed onto 2 × 8 mm wicks cut from Whatman 3μ chromatography paper (Whatman International, Maidstone, UK). Allozyme variation was determined via horizontal starch-gel electrophoresis. Gel (11%) buffers and stain recipes followed Soltis *et al.* (1983).

Ten enzyme systems stained for 22 putative loci found to be informative within the genus. System 7 (Soltis *et al.*, 1983) was used to resolve

triosphosphate isomerase (Tpi-1, Tpi-2), aspartic acid transaminase (Aat-1, Aat-2, Aat-3, Aat-4), and phosphoglucoisomerase (Pgi-2). System 9 was used to resolve malate dehydrogenase (Mdh-1, Mdh-2, Mdh-3, Mdh-4), 6-phosphogluconate dehydrogenase (6-Pgd-1), and phosphoglucomutase (Pgm-1, Pgm-2). System 11 was used to resolve aconitase (Aco-1, Aco-2), diaphorase (Dia-1, Dia-2, Dia-3, Dia-4), isocitrate dehydrogenase (Idh-1), and shikimic dehydrogenase (Skdh-1).

Analysis of spores.—Spore sizes were measured as the longest diameter of a minimum of 20 spores from each of ten plants and compared with published values for *Botrychium ascendens* and related diploid species.

Morphological comparisons.—Leaf morphology for Newfoundland plants was compared with *Botrychium ascendens* from western states and with *B. campestre* and *B. lineare* from all sites listed in Table 1. Illustrations were prepared to capture the range of morphology for each species.

Results

Allozyme electrophoresis.—Twenty enzyme loci were variable among the compared taxa (Table 2). The allelic composition of *Botrychium ascendens* from Newfoundland was identical to the combined expression of western *B. ascendens* populations from Oregon, Washington, Montana, Nevada, California and Alaska (Table 2). Plants of identical genotype were present within the Newfoundland populations and the type locality of *B. ascendens* in Oregon. *Botrychium ascendens* from all sites displayed fixed heterozygosity at seven of the 22 loci examined. Four additional loci varied among populations for fixed heterozygosity, with some plants expressing only one of the alleles of the heterozygous condition. At all loci, the alleles present in *B. ascendens* are also present in *B. lineare*, *B. campestre* and/or *B. crenulatum*. At five loci, *Tpi-1*, *Tpi-2*, *Mdh-1*, *Mdh-2*, and *Skdh-1*, the single allele present in the American genotype of *B. lunaria* (L.) Swartz is not present in *B. ascendens*.

Analysis of spores.—Average spore size of *Botrychium ascendens* plants from Newfoundland was 41 μm and ranged from 39 to 44 μm. Wagner and Wagner (1986) reported spore sizes of 44–47 μm for *B. ascendens* from western plants. Wagner and Wagner (1986) reported a range of 34–38 μm for *B. campestre*. Wagner and Wagner (1994) did not include spore size in their description of *B. lineare*. Our measurements of spores of *B. lineare* averaged 36.2 μm and ranged from 35 to 39 μm.

Morphological comparisons.—Leaf morphology for Newfoundland plants was compared with *Botrychium ascendens* from western states and with *B. campestre* and *B. lineare*. Small Newfoundland plants closely resemble *B. lineare* and *B. campestre* (Figs. 1–3). Larger plants display features more typical of western plants of *B. ascendens* (Figs. 4–5). Typical plants of *B. ascendens* display a morphology intermediate between *B. campestre/lineare* and *B. crenulatum* (Fig. 6).

TABLE 2. Allozyme composition of *Botrychium ascendens* and putative parents. The number of individuals of each species analyzed is indicated (in parentheses). Alleles for each locus are represented by numbers. Numbers separated by a comma are alternate alleles expressed in different individuals. Numbers joined by "+" are two alleles present (fixed) in all individuals of the species or population. In *Aco*, *Idh*, and *Pgi*, *B. ascendens* displays fixed heterozygosity in most populations, but in some populations displays only one of the alleles present in the heterozygous state.

Allozyme locus	*B. campestre* (197)	*B. lineare* (91)	*B. ascendens* (186)	*B. crenulatum* (168)	*B. lunaria* (583)
Aat-1	2	2	1 + 2	1, 2	2
Aat-2	3	3	3	3	3
Aat-3	2	2, n	2	n	n
Aat-4	3	3	2 + 3	2, 3	2, 3
Aco-1	2	2	1, 1 + 2	1, 2	1
Aco-2	1	3	3, 1 + 3	1, 3	1, 3
Dia-1	4	2, 4	2 + 4	2	2
Dia-2	1	1	1	1	1
Dia 3	2, 3	2, 3	2 + 3	3	3
Dia-4	4	4	4	n	n
Idh	2, 3	1, 2	2, 2 + 3, 3, 2 + 4	3, 4	2
Mdh-1	3	1, 3	1 + 2	2	4
Mdh-2	3	3	2 + 3	2	5
Mdh-3	2*	1*, 2*	2*	2*	2
Mdh-4	2	2	2	n	n
6Pgd	1	1	1 + 5**	5	5
Pgi-2	2	2	2, 2 + 4, 4	4, 5	4
Pgm-1	1***	1***	1***	1	1
Pgm-2	2, 4	2	2	2	2
Skdh	1, 2	1	1	1	2
Tpi-1	3	3	3	3	4
Tpi-2	3	3	3	3	4

*In these species an additional spot of unknown origin always trails the principal allelic spot at a uniform distance regardless of the allele.
**Activity of the homodimers varied among populations; in some, only the heterodimer expressed normal activity.
***In these species an additional spot, possibly from a locus duplication, always precedes the principal allelic.
n = null, i.e., no allele expressed.

DISCUSSION

Moonwort ferns of *Botrychium* subgenus *Botrychium* are notoriously difficult to identify with certainty by morphological characters alone. They have been appropriately referred to as cryptic species (Hauk and Haufler, 1999). Because of their small size and simple morphology, differences between species are subtle and tend to be statistical rather than absolute. This problem is compounded in allotetraploids, in which the ranges of morphological characters overlap those of the parental diploids.

In contrast to morphology, genetic markers clearly define species of moonworts and, for allotetraploids, provide evidence of the ancestral diploid

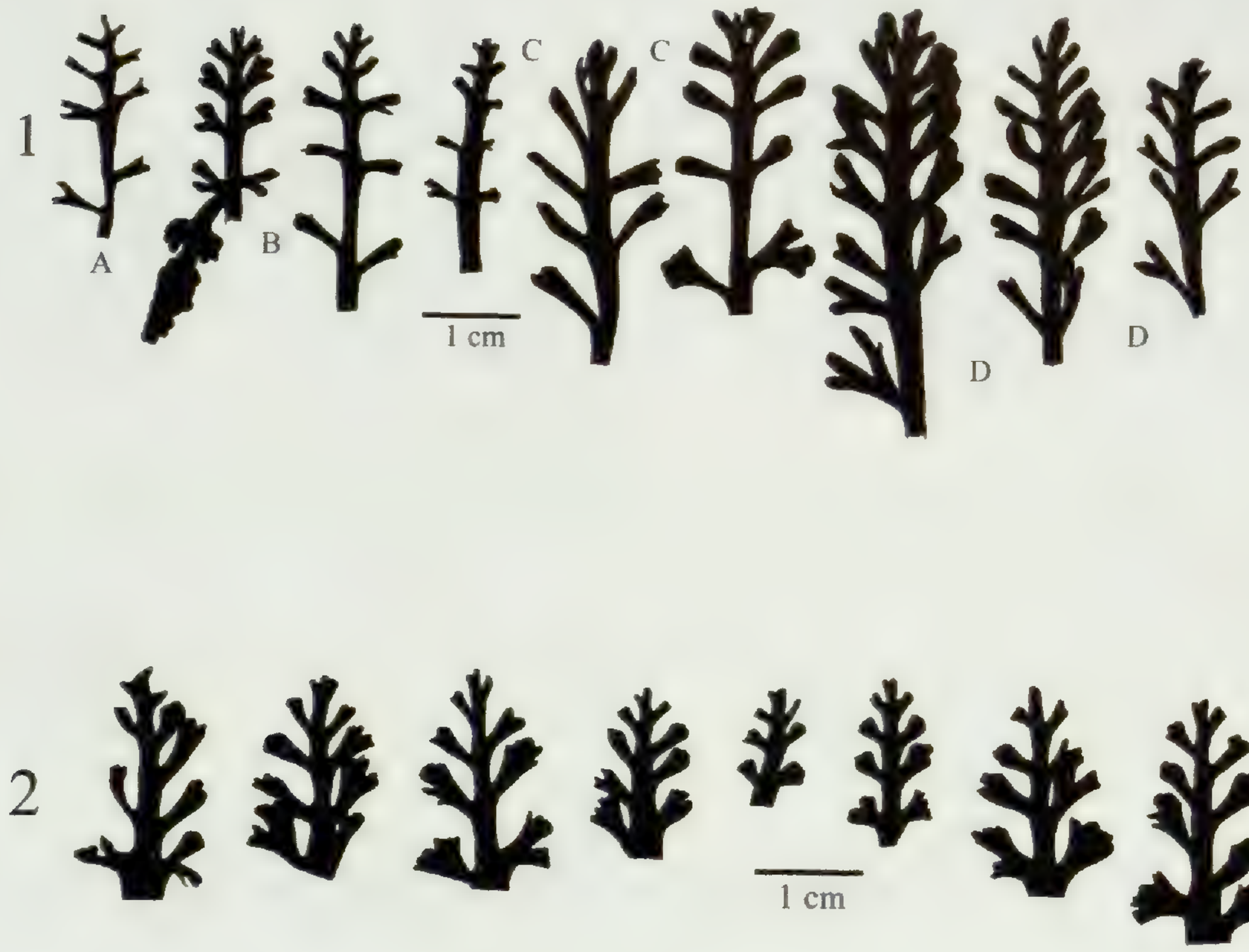

FIG. 1. *Botrychium lineare* trophophores, showing typical slender pinnae with margins essentially straight or narrowing to base, notched or forked at tip. A. Washington (*Kirk 200 et al.* WTU). *B.* Type locality, Oregon (*Farrar 3918, E2235* ISC). C. Oregon (left to right: *Zika 12906* OSC; *Wagner 81128 & Wagner* MICH; *Zika 11353A* OSC). D. Colorado (left to right: *Farrar 3794, 3797, 3801* ISC).
FIG. 2. *Botrychium ascendens* trophophores. Population variation showing narrow extremes from

species involved in their formation. In our comparison of genetic profiles determined for 22 loci in 10 enzyme systems, plants of *B. ascendens* from Newfoundland were consistent with those obtained for western plants from throughout the range of *B. ascendens*, including the type locality in northeastern Oregon. They differed from some (but not all) western populations only in not possessing fixed heterozygosity at *Aco-2*. This character state is also present in some populations in Oregon and Alaska. As with all plants of *B. ascendens*, in addition to possessing fixed heterozygosity, Newfoundland plants differed from both *B. lineare* and *B. campestre* in possessing many alleles not present in either of those species.

Morphological comparison of the Newfoundland *Botrychium ascendens* with *B. campestre* and *B. lineare* is useful in field and herbarium identifications. Slender plants from Newfoundland with narrow pinnae resemble *B. lineare* (Fig. 1), but tend to have broadened distal segments with more dentate outer margins (Fig. 2). The larger Newfoundland plants (Fig. 4) compare favorably with western specimens of *B. ascendens*, including plants from the type locality. They show somewhat more highly divided segments than the type collection of *B. ascendens* from Wallowa Co., Oregon (*W. H. Wagner 83363 et al.* MICH). However, this variation in morphology is comparable to larger specimens collected subsequently from the type locality, as well as material collected in Washington and Alaska (Fig. 5). There also exist many individuals (not illustrated) with morphology intermediate and transitional between the plants depicted in Fig. 2 and Fig. 4 from Newfoundland, showing increasingly broad and fan-shaped proximal pinnae. Thus, as the plants increase in size, their morphology trends away from the characteristic narrowness of *B. lineare*.

In comparison to *Botrychium campestre*, *B. ascendens* has less fleshy central axes and has basal pinnae that are more uniformly and broadly fan-shaped. Pinnae of *B. ascendens* are also more evenly spaced along the rachis of the trophophore and are more regularly and more deeply cleft into spreading lobes (Figs. 2, 4, 5). *Botrychium campestre* (Fig. 3) sometimes shows partial fusion of adjacent pinnae, and a broadly decurrent basal margin to some of the basal pinnae, features absent in *B. ascendens*. The outer margins of pinnae and pinnae lobes are dentate in *B. ascendens* and usually entire to crenulate in *B. campestre*. The basal pinnae are usually the largest in *B. ascendens* and

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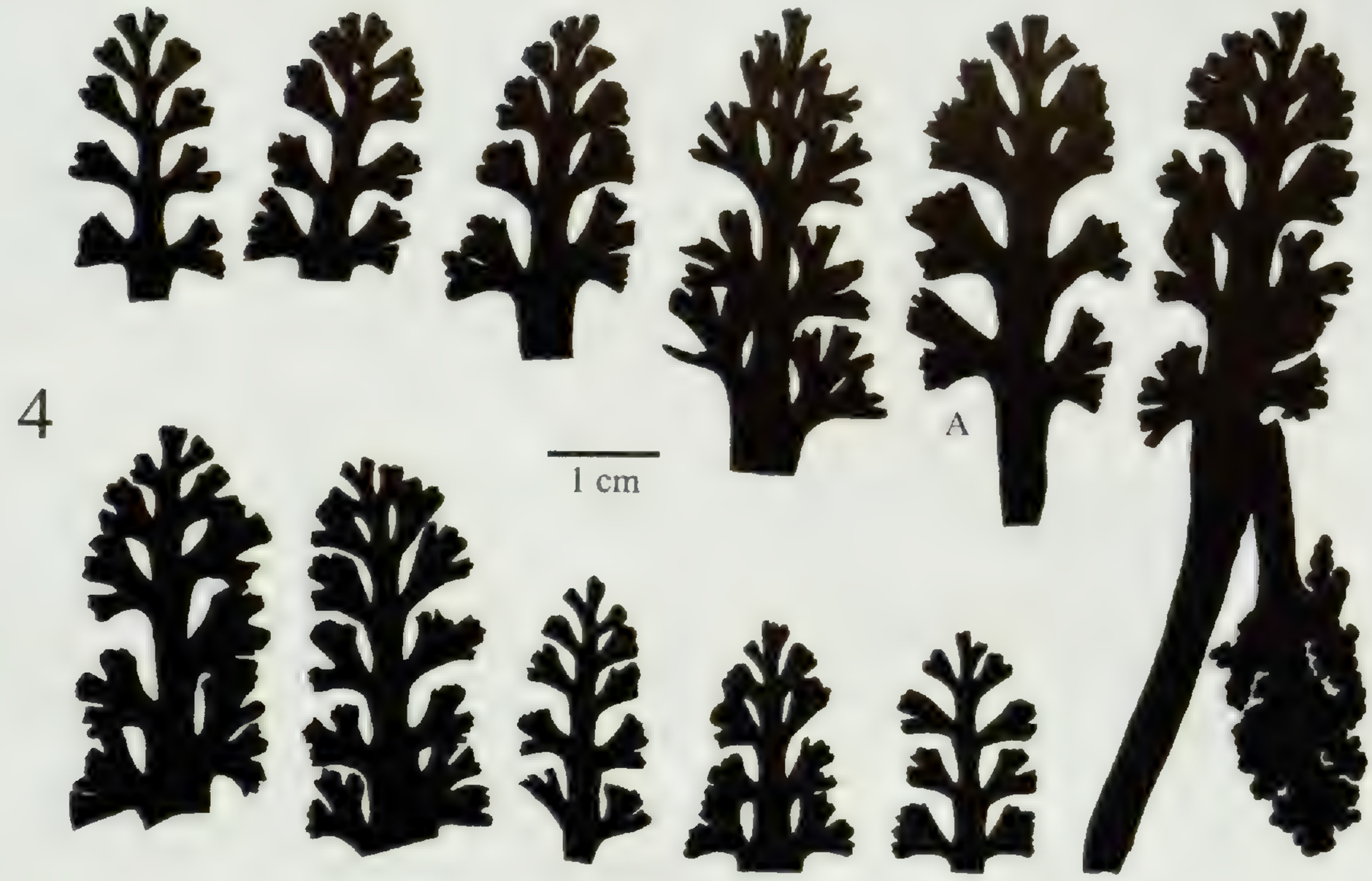
small plants in Newfoundland. Distal pinnae slender, as in *B. lineare*, but proximal pinnae broadened and with outer margins tending to be dentate (*Zika 16328* WTU).

FIG. 3. *Botrychium campestre* trophophores. Larger plants showing fleshy rachis, decurrent base of proximal pinnae, irregular and coalescing pinnae. Some smaller plants with more linear segments, approaching *B. lineare*, but pinnae apices more irregular. A. Michigan (*Wagner 85024 et al.* ISC). *B.* Michigan (*Wagner 85025 et al.* MICH). C. Michigan (*Wagner 85026 et al.* MICH). D. Michigan (*Wagner 85024 et al.* MICH). E. Minnesota (*Farrar 3495* ISC). F. Nebraska (*Farrar 86-6-2-3D* ISC). G. Minnesota (*Farrar 3497* ISC). H. Iowa (*Farrar 952* ISC). I. Type locality, Iowa (*Farrar 3484* ISC, on left; *Farrar 86-5-31-1* ISC).



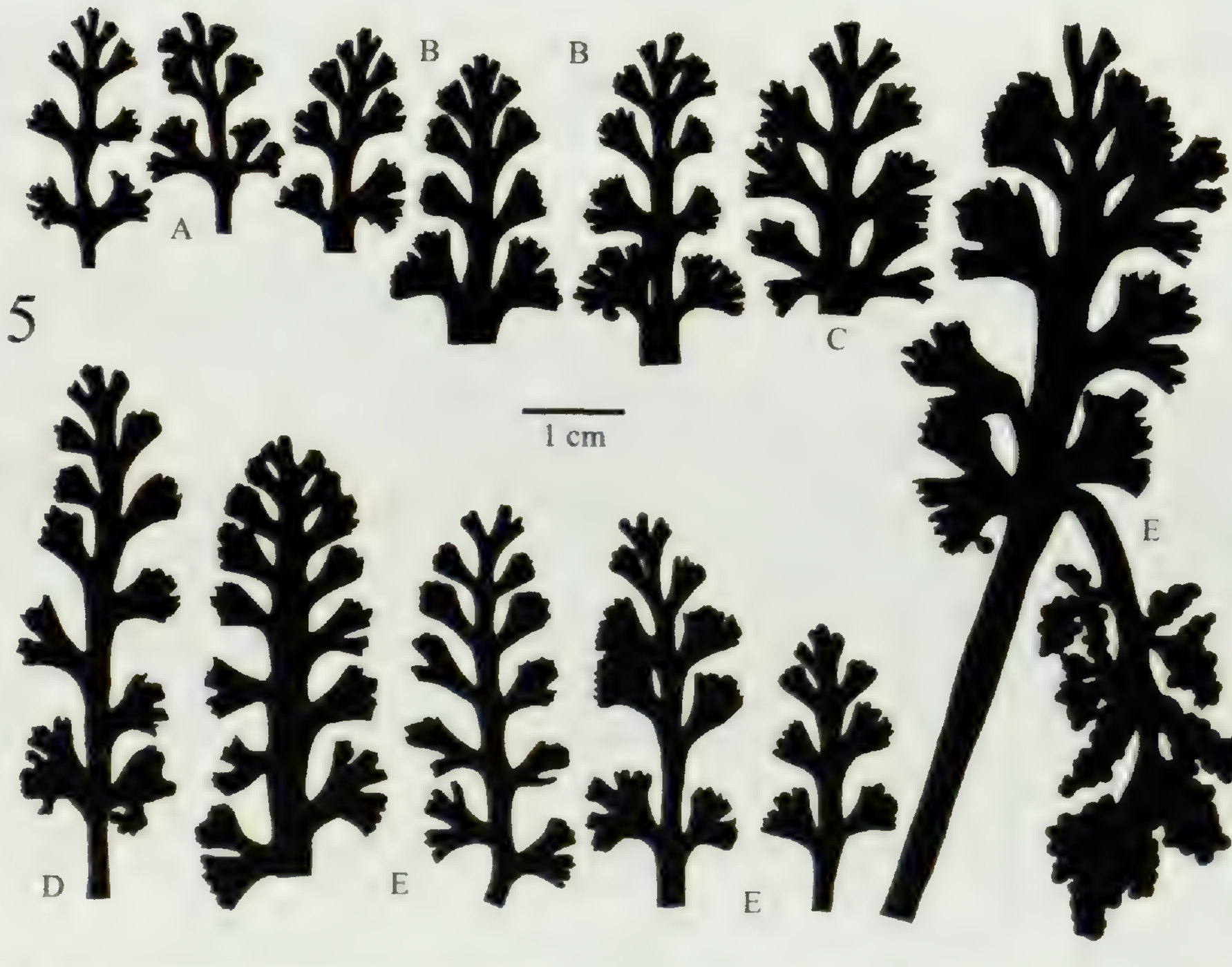


FIG. 4. *Botrychium ascendens* trophophores. Population variation showing large and deeply divided pinnae from Newfoundland. A. *Britton 10671 & Anderson* (MICH); all others *Zika 16328* (WTU).
FIG. 5. *Botrychium ascendens* trophophores. Typical plants from western North America. A. Type collection, Oregon (*Wagner 83363 et al.* MICH). *B.* Type locality, Oregon (*Zika 17090* OSC). C. Washington (*Larson 254* WTU). D. Alaska (*Smith s.n.* ALA). E. Washington (*Buege s.n.* WTU).

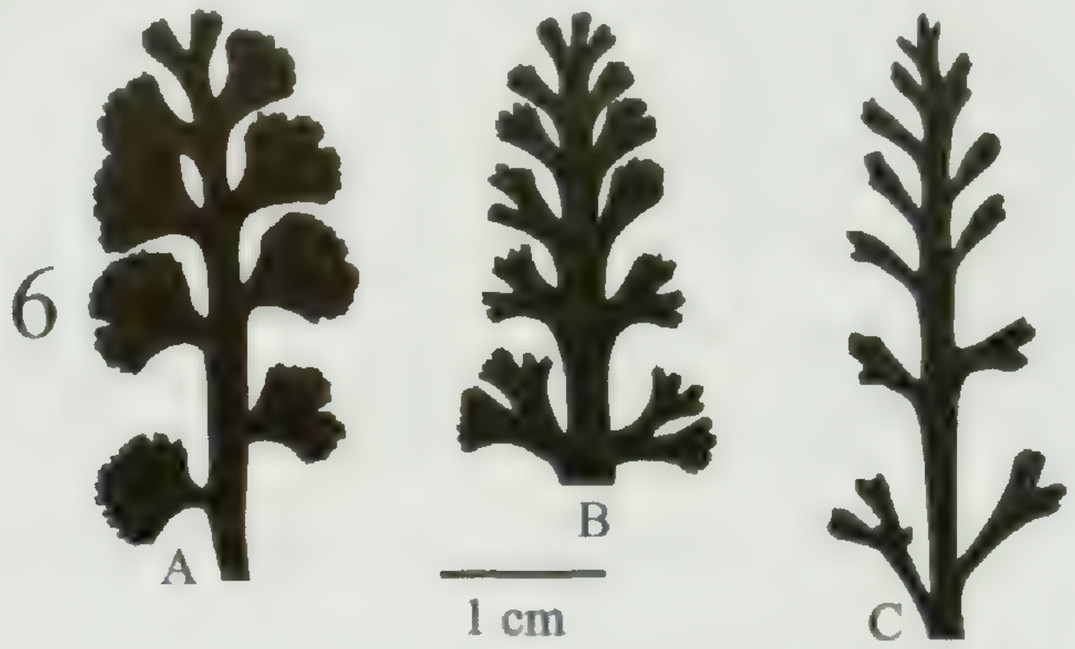


FIG. 6. Proposed allopolyploid parentage of *Botrychium ascendens*. A. *Botrychium crenulatum* (*Farrar 2031* ISC). B. *Botrychium ascendens* (*Farrar 1818* ISC). C. *Botrychium lineare* (*Farrar 3921* ISC).

frequently bear a few sporangia, whereas a non-basal pair is usually largest in *B. campestre*, and trophophore pinnae seldom bear supernumerary sporangia. Some *B. campestre* show basal pinnae erect in the axil between trophophore and sporophore, a feature not observed in *B. ascendens*. In large, well-developed plants, the non-basal pinnae of *B. ascendens* often tend to have a broader outer margin.

An additional feature helpful in separating *Botrychium ascendens* from *B. campestre* and *B. lineare* is the length of the sporophore stalk, which, in *B. ascendens*, reaches 1/3 to 2/3 the length of the trophophore. In *B. campestre* and *B. lineare* the sporophore stalk is usually ¼ or less the length of the trophophore. It must be noted, however, that this character is useful only in mature plants when (at spore release) the sporophore stalk has ceased elongation. The diploid species, *B. campestre* and *B. lineare*, also have smaller spores (34–39 μm) than does tetraploid *B. ascendens*, from Fogo Island and elsewhere (39–47 μm). All three can bear inconspicuous asexual reproductive gemmae on their subterranean stems (Johnson-Groh *et al.*, 2002). Both *B. campestre* and *B. lineare* have been reported from northeastern North America (Kartesz, 1999; Hinds, 2000).

Recombinational heterozygosity at a given locus segregates in meiosis and recombines in syngamy to produce a predictable proportion of individuals in a population that are homozygous. Occurrence of heterozygosity in all individuals of a population at a large proportion of loci is best explained as non-recombinational or fixed heterozygosity resulting from allopolyploidy. Plants of *B. ascendens* from Newfoundland and elsewhere display identical fixed heterozygosity at seven of 22 loci examined, and, in some populations, at four additional loci. Consistent with its chromosome number of n = 90 (Wagner and Wagner, 1986), this strongly indicates that *B. ascendens* is an allotetraploid derived through ancient hybridization between two diploid species. This is also supported by morphological evidence.

The allelic composition of *Botrychium ascendens* matched an expected composition resulting from hybridization between either *B. campestre* or *B. lineare* and *B. crenulatum* (Table 2). Of the 11 loci displaying only a single

allele in *B. ascendens*, this allele is present in both of these putative parental lines (8) or null in *B. crenulatum*. In the loci displaying fixed heterozygosity, *B. crenulatum* possesses at all loci the alleles necessary to create, in combination with *B. campestre/lineare*, the allelic combinations present in *B. ascendens*. This includes an allele at *Mdh-2* that, among diploid species of *Botrychium*, is found only in *B. crenulatum*. Close genetic similarity between *B. lineare* and *B. campestre* does not allow differentiation between these two species as to the most likely parent, but the *B. campestre/lineare* lineage is strongly implicated.

Botrychium lunaria is the only diploid species other than *B. crenulatum* possessing the broad pinna shape predicted for the non-*B. campestre/lineare* parent of *B. ascendens*. The American genotype of *B. lunaria* is homozygous at five loci (*Tpi-1*, *Tpi-2*, *Mdh-1*, *Mdh-2*, *Skdh-1*) for alleles not present in *B. ascendens*, and fails to provide the complimentary allele missing from *B. campestre/lineare* at an additional locus (*Aat-1*). The European genotype of *B. lunaria* is similar to that of *B. crenulatum*, but does not contain the *Mdh-2* allele uniquely present in *B. crenulatum* and *B. acendens* (Stensvold 2008).

The morphology of *Botrychium ascendens* is also consistent with parentage of *B. crenulatum* and *B. lineare* (Fig. 6). The ascending pinnae and their tendency to be bifurcate likely reflect tendencies inherited from *B. lineare*. The dentate outer margins of *B. ascendens* pinnae reflect the intermediacy between the crenulate margins of *B. crenulatum* and the entire margins of *B. lineare*.

Botrychium lineare and *B. campestre* occur in both eastern and western North America. *Botrychium crenulatum* is known only in western North America, ranging in western mountains from southern California and Nevada to southern British Columbia and eastward to central Alberta. If these patterns represent distributions of the parent species at the time of the formation of *B. ascendens*, eastern Canadian populations of *B. ascendens* likely result from migration of the species from western to eastern North America. *Botrychium ascendens* is a fertile tetraploid. Gametophytes from a single spore are capable of producing sporophytes through self-fertilization in *Botrychium*, thus long distance migration via single spores is possible. The habitat of the plants in Newfoundland is remarkably similar to the back-beach sand dune habitat of *B. ascendens* and other *Botrychium* species in south coastal Alaska. A single collection of *B. ascendens* from the south shore of Hudson Bay (*Moir 1444* CAN) suggests the possibility of a broader occurrence of *B. ascendens* in similar habitats across northern Canada.

Both morphological and genetic evidence confirm that *Botrychium ascendens* is extant in the province of Newfoundland, in Fogo District, Fogo Island, off the northeast shore of the island of Newfoundland.

We hope this discovery in Newfoundland will encourage botanists in eastern Canada to search for additional extant populations of *Botrychium ascendens*, a small and inconspicuous species that possibly has been overlooked in coastal and other habitats.

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Spore Maturation and Release of Two Evergreen Macaronesian Ferns, *Culcita macrocarpa* and *Woodwardia radicans*, along an Altitudinal Gradient

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Abstract.—The variables affecting spore phenology have been poorly studied in contrast with the abundant literature on leaf phenology. This paper deals with the influence of altitude and canopy cover on spore maturation and release of *Culcita macrocarpa* and *Woodwardia radicans* in the island of São Miguel, Azores. The study was conducted during one sporing season at three altitudes (400, 600, and 800 m). In both species spore maturation occurred in autumn and may be controlled by the previous accumulation of photosynthates. Spores were not released until late winter owing to a requirement for dry weather conditions. Dispersal took place later at higher altitude, due to lower temperature and higher humidity. This gradual liberation of spores along an altitudinal gradient is important for the endemic Azores bullfinch *Pyrrhula murina* (a bird that feeds on spores in winter), providing food over an extended period.

Key Words.—Azores, altitudinal gradient, spore phenology, laurel forest, *Culcita*, *Woodwardia*, *Pyrrhula*, Blechnaceae, Culcitaceae

Diaspore (seed or spore) maturation and dispersal play a key role in plant population dynamics. Diaspore dispersal is a prerequisite for the establishment of new populations and a vehicle for gene flow between populations (Haufler, 2002). The selective forces that influence the timing of flowering, fruiting and seed dissemination have been widely investigated (Fenner, 1998). In contrast, few studies have dealt with fern spore maturation and dispersal (e.g., von Aderkas and Green, 1986; Durand and Goldstein, 2001; Sawamura *et al.*, 2009). Spore production is affected by several environmental factors, such as temperature, humidity and canopy cover (Odland, 1998; Greer and McCarthy, 2000; Arens, 2001).

Altitudinal gradients are powerful 'natural experiments' for testing ecological and evolutionary responses of organisms to abiotic factors. There are two categories of environmental changes with altitude: those physically tied to meters above sea level, such as temperature; and those that are not generally

altitude specific, such as hours of sunshine (Körner, 2007). Altitudinal ecological gradients reflect differences in genetic (Herrera and Bazaga, 2008), vegetative (Scheidel and Bruelheide, 2004), reproductive (Dangasuk and Panetsos, 2004) and phenological traits (Schuster *et al.*, 1989). In some fern species, spore production decreases toward higher and lower altitudinal distribution limits (Sato *et al.*, 1989; Odland, 1998).

We studied the influence of altitude on spore maturation and dispersal of *Culcita macrocarpa* C. Presl. (Culcitaceae) and *Woodwardia radicans* (L.) Sm. (Blechnaceae). These ferns occur in a warm-temperate range that extends discontinuously through Macaronesia (Azores, Madeira and Canary islands), the Atlantic coast of the Iberian Peninsula and, in the case of *W. radicans*, some locations in the Mediterranean region. Both species are considered relicts of the tropical flora that covered the Mediterranean area during the Tertiary period (Pichi-Sermolli, 1979). *Culcita macrocarpa* and *W. radicans* share the same life-form, with large shoots that grow above ground and evergreen leaves over two meters long, which makes them the largest ferns in Europe.

Culcita macrocarpa and *W. radicans* abound in the Azores (Dias, 1996), the wettest and northernmost Macaronesian archipelago, where they occur along a large altitudinal gradient of 300–1000 m for *C. macrocarpa* and 50–950 m for *W. radicans* (Schäfer, 2002). This makes the Azorean populations a suitable model to study the effects of altitude-correlated environmental factors on spore maturation and release. Additionally the sporangia of these two species are important winter food resources (Ramos, 1995, 1996a) for the critically endangered Azores bullfinch (*Pyrrhula murina* Godman). This bird is restricted to about 6000 ha in the east of the island of São Miguel, from which only 1675 ha correspond to native forest, largely invaded by exotic species (Ramos, 1996b; Ceia, 2008). We compared the spore phenology traits of *C. macrocarpa* and *W. radicans* at three altitudes in São Miguel island. Our specific questions were: (1) What are the effects of altitude on temperature, humidity and vegetation cover? (2) Is the timing and success of spore maturation influenced by temperature, humidity and vegetation cover? (3) Does the altitudinal gradient affect the dates of spore release? (4) What are the implications for the conservation of the Azores bullfinch?

Materials and Methods

Study sites.—The study was conducted in Serra da Tronqueira, São Miguel Island, archipelago of the Azores (37°47′N, 25°13′W). This area is a steep volcanic range with oceanic climate (Marques *et al.*, 2008). Temperatures are mild throughout the year (mean annual temperature 17°C at sea level) and there is no frost. Yearly rainfall increases with altitude varying from ~1500 mm at sea level to >3000 mm at highest altitudes (~1100 m). The canopy of the natural laurel forest is dominated by evergreen trees and shrubs [*Erica azorica* Hochst. ex Seub., *Frangula azorica* V. Grubov, *Ilex perado* Aiton ssp. *azorica* (Loes.) Tutin, *Juniperus brevifolia* (Seub.) Antoine, *Laurus azorica* (Seub.)

Franco, *Myrsine africana* L., *Prunus lusitanica* L. ssp. *azorica* (Mouillef.) Franco, *Vaccinium cylindraceum* Sm. and *Viburnum tinus* L. ssp. *subcordatum* (Trel.) P. Silva]. Most of the original forest has been converted to plantations of *Cryptomeria japonica* (L. fil.) D. Don (300–900 m) or invaded by alien species: *Hedychium gardnerarum* Sheppard ex Ker-Gwal. (0–950 m), *Clethra arborea* Aiton (500–900 m) and *Pittosporum undulatum* Vent. (50–650 m) (Schäfer, 2002). Ferns are rare to absent in patches of dense homogenous exotic vegetation.

To study environmental and spore phenology variables (maturation and release), we selected three sites with both *C. macrocarpa* and *W. radicans* at 400, 600 and 800 m (hereafter referred to as low, mid and high altitude, respectively). The low altitude site was a laurel forest densely invaded with *P. undulatum* and *Acacia melanoxylon* R. Br., whereas, the mid altitude site was a laurel forest moderately invaded by *C. arborea*. The high altitude site was a laurel forest mixed with *Pinus nigra* Arn. and *C. japonica* plantations. At each altitude 12 mature individuals (i.e., with at least one fertile leaf) of each species were randomly selected and tagged, yielding a total of 72 marked individuals (12 individuals × 3 altitudes × 2 species).

Environmental variables.—Temperature and relative humidity measures were obtained with a thermohigrometer (HOBO Pro v2 logger, Onset Computer Corporation, USA) at each altitude (400, 600 and 800 m). These thermohigrometers were placed 1.5 m above the ground under tree canopy. Data were hourly recorded for one year starting in April 2007. To determine canopy cover, hemispherical photographs at 1.3 m over each tagged individual fern were taken using a digital camera (Nikon CoolPix 995, Nikon, Japan) with a fish eye converter (FC-E8, Nikon, Japan). Photos were orientated to the magnetic north and horizontally located using a bubble level (Valladares, 2006). Images were processed with Gap Light Analizer 2.0 (Forest renewal BC, Canada). Canopy cover (%) was calculated as 100 − canopy openness (%), the later being percentage of open sky seen from beneath a forest canopy.

Spore phenology variables.—From 30 October 2006 to 15 May 2007, the six study populations were visited every ca. 10 days to assess whether timing of spore maturation and release differed with altitude. At the base of a fertile pinna of each tagged individual, two opposite pinnules were marked, one to study spore maturation and the other to study spore release. Maturation was studied by collecting six sori per pinnule in each visit until the beginning of spore release (9 February 2007). Sori were stored in Eppendorf vials to keep sporangia hydrated and avoid spore release. In the laboratory, sporangia were opened with a lancet and their content was observed with a light microscope. A random sample of 400 spores per individual were sorted into three morphological groups: mature, immature and aborted. Mature spores have a two-layered wall, with both perispore and exospore, and their protoplast is fulfilled with lipid drops (Tryon and Lugardon, 1991). Immature spores lack a perispore and oil drops and aborted spores lack a protoplast and/or are collapsed. Spore maturation date was defined as the number of days since January 1 (i.e., Julian days) until an individual possessed 90% mature spores.

The percent of aborted spores was determined from the spore sample of the visit preceding the spore release date of each individual (see below).

Indusia opening was used to estimate the timing of spore release. Both studied species have indusia that completely enclose the sori and as soon as indusia open, most spores are released out of the sori (pers. observation). During each visit we counted on the marked pinnules the number of sori with open indusia. Spore release date was defined as the number of Julian days until 50% of an individual's indusia were open.

Statistical analyses.—Generalized Linear Models (GLMs; McCullagh and Nelder, 1989) were built for the following variables: canopy cover percentage, spore maturation date, spore release date and spore abortion percentage using the GENMOD procedure of SAS 9.0 (SAS Institute, 2002). GLMs were used because these variables departed from the normal distribution. A binomial distribution with logit link function was used for canopy cover percentage and abortion percentage, and a Poisson distribution with log link for maturation date and release date because under these conditions the explained variation was maximal. The explanatory variables considered in the models were fern species (*C. macrocarpa* and *W. radicans*) and altitude (low, mid and high); canopy cover percentage was included as a covariate in the maturation date and release date models. All of these variables were considered as fixed effects. Subsequent pairwise comparisons were made using LSMEANS statement of SAS 9.0 (SAS Institute, 2002). The relationship between maturation date and release date was assessed by Spearman's rank correlation coefficient within each species. This analysis was performed with SPSS 13.0 (SPSS, 2003). Data are shown as mean ± SE unless otherwise specified.

RESULTS

Environmental variables.—Temperature decreased with increasing altitude. Yearly means were 15.5°C, 13.6°C, and 12.4°C in the low, mid and high altitudes, respectively. The average altitudinal gradient was −0.78°C/100 m [= (12.4°C–15.5°C)/(800 m–400 m)]. Temperatures were mild throughout the year (Fig. 1A), with only 7°C difference between the warmest (July, August) and the coldest (February, March) months in the three altitudes (Fig. 1A). The winter was frost free, with absolute minimums above 4°C. Relative humidity increased with increasing altitude (Fig. 1B). In the three altitudes monthly means were generally far above 85% although humidity decreased during spring and summer, with absolute minimums below 40%. Canopy cover percentage differed significantly among altitudes but not between fern species (Table 1, Fig. 2). Cover increased in the order: mid < low < high, with 63% ± 2, 71% ± 3 and 83% ± 1, respectively (data for both fern species pooled).

Spore maturation and abortion.—At the beginning of the study (October 30) *C. macrocarpa* had greater than 70% mature spores at the three altitudes (Fig. 3). *Woodwardia radicans* showed a similar percentage at low altitude, whereas percentages were lower at mid and, especially, low altitudes. This initial difference among altitudes disappeared with time and maturation date,

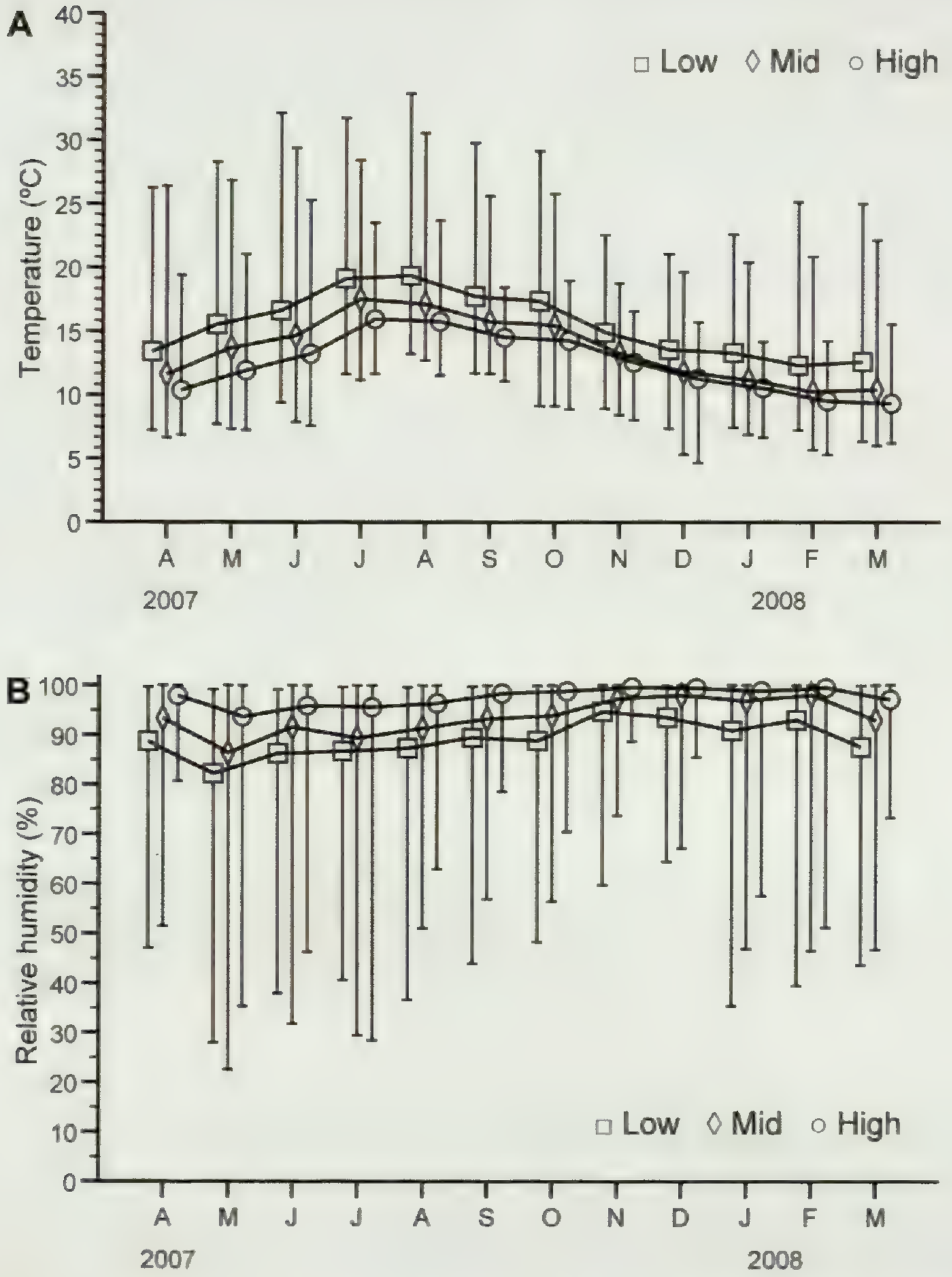

FIG. 1. Monthly temperature (A) and relative humidity (B) at the three study altitudes (mean ± absolute maximum and minimum). Climatic data were recorded hourly with thermohigrometers placed 1.5 m above the ground under tree canopy. Bars are the highest and lowest value in each month.

i.e., the number of days before reaching 90% mature spores, was not significantly affected by altitude (Table 1). Canopy cover also did not have a significant effect on maturation date. Differences between species were significant, with earlier maturation in *C. macrocarpa* [315 Julian days (= November 11) ± 4 days, data from the three altitudes pooled] than in *W. radicans* [345 Julian days (= December 11) ± 6 days]. Neither species reached 100% mature spores at the end of the study period (Fig. 3). This was mainly due to existence of some aborted spores in all study individuals. Abortion percentage did not differ significantly between species or among sites (Table 1). Both species had low abortion percentages at the three altitudes (means ≤ 8%).

TABLE 1. Summary of GLMs for the effects of species (*Culcita macrocarpa* or *Woodwardia radicans*) and altitude (low, mid or high) on canopy cover percentage, spore maturation date, spore abortion percentage and spore release date. Canopy cover percentage was considered as a covariate in the models for maturation date and release date. Significant values are in bold. d.f. = degrees of freedom.

Variable	Effect	d.f.	χ^2	*P*
Canopy cover (%)	species	1	0.01	0.908
	altitude	2	54.02	**<0.0001**
	species × altitude	2	1.16	0.559
Maturation date	species	1	12.16	**0.0005**
	altitude	2	5.28	0.071
	canopy cover	1	0.49	0.483
	species × altitude	2	4.86	0.088
Abortion (%)	species	1	0.01	0.922
	altitude	2	5.02	0.081
	species × altitude	2	5.66	0.059
Release date	species	1	0.66	0.416
	altitude	2	6.11	**0.047**
	canopy cover	1	0.38	0.536
	species × altitude	2	0.06	0.968

Spore release.—In both species, spore release started in mid-January and ended in early May (Fig. 4). Spore release of each individual was gradual, and for *C. macrocarpa* highly synchronous among individuals at all altitudes, as revealed by the small standard errors. *Woodwardia radicans* was less synchronous at high altitude. Release date (number of days before reaching 50% indusia open) was only significantly affected by altitude (Table 1).

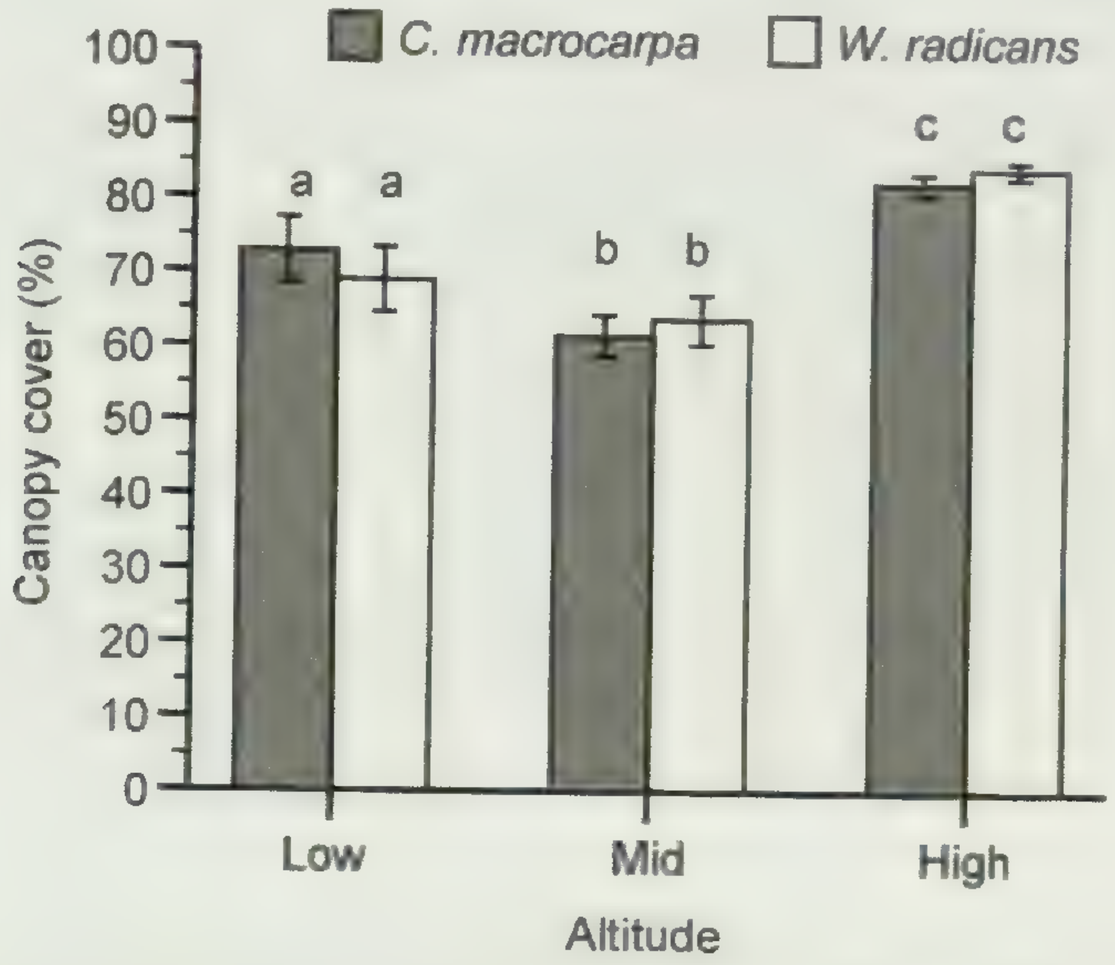

FIG. 2. Percent canopy cover (mean ± SE) for *Culcita macrocarpa* and *Woodwardia radicans* at three altitudes. Each mean represents twelve hemispherical photographs taken over each tagged individual (n = 12). Different letters indicate significantly different means ($P < 0.05$, LSMEANS; SAS Institute, 2002).

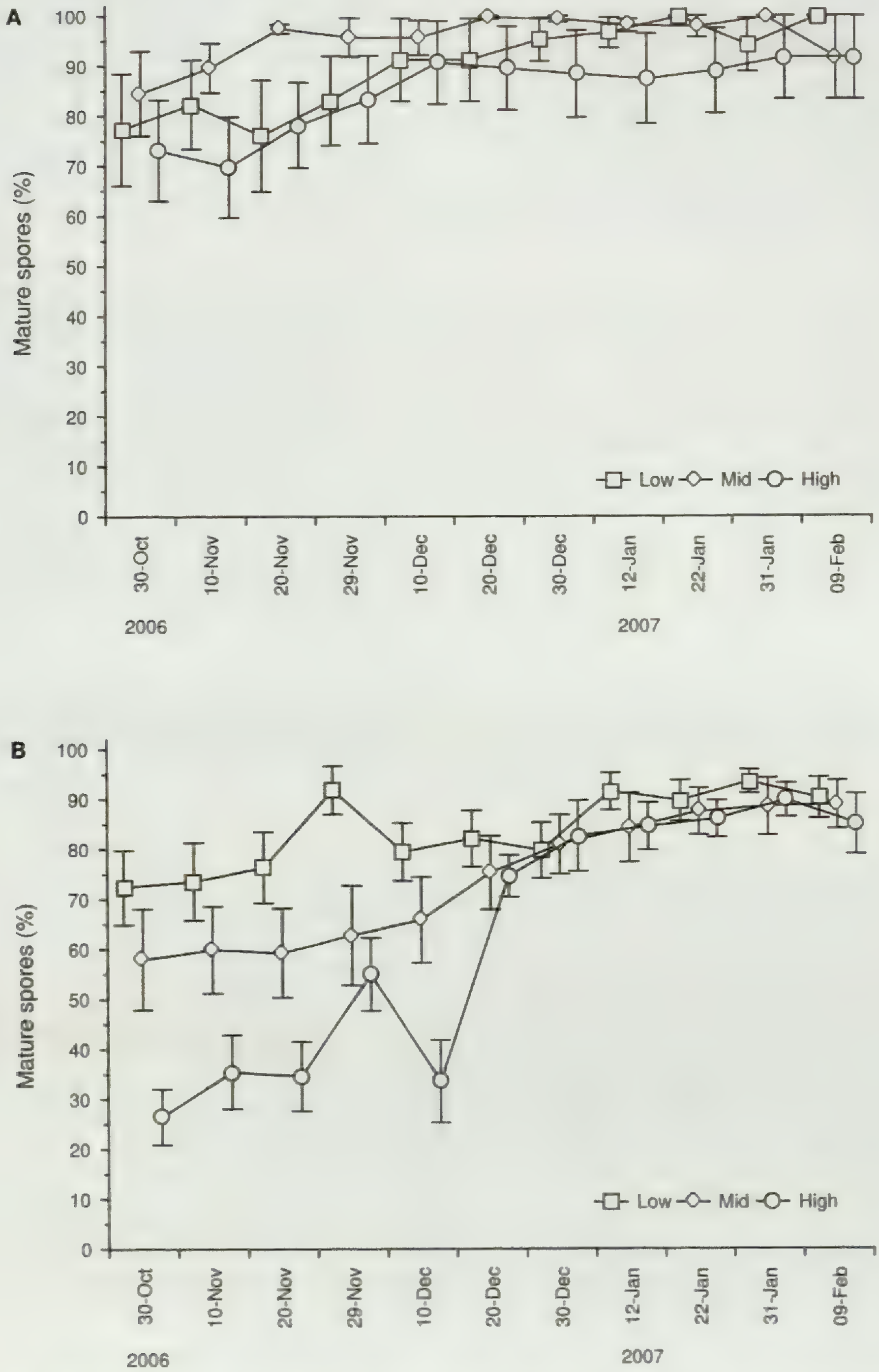

FIG. 3. Seasonal changes in spore maturation (percent mean ± SE) for *Culcita macrocarpa* (A) and *Woodwardia radicans* (B) at three altitudes. Each mean represents twelve permanently marked individuals (n = 12). Four hundred spores per individual plant were observed for each date.

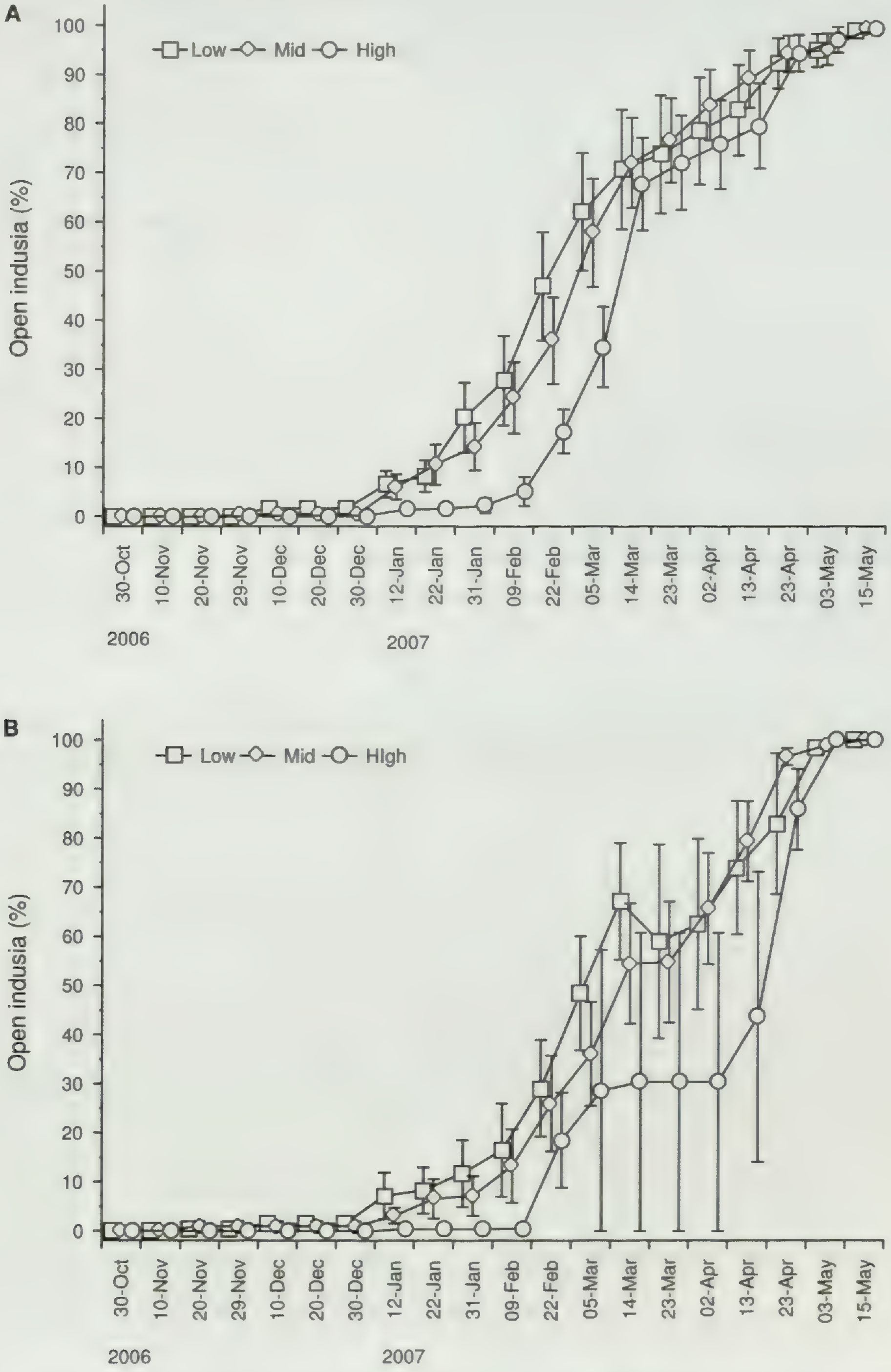

FIG. 4. Seasonal changes in opening of indusia (percent mean ± SE) for *Culcita macrocarpa* (A) and *Woodwardia radicans* (B) at three altitudes. Each mean represents twelve permanently marked individuals (n = 12). One fertile pinnule per individual was observed.

Release took place earlier at low altitude [54 Julian days (= February 23) ± 8 days, data of both species pooled] than at high altitude [80 Julian days (= March 21) ± 5 days], whereas release date at mid altitude [72 Julian days (= March 13) ± 6 days]) was not significantly different from those at high and low altitudes ($P > 0.05$, LSMEANS). Release date showed a significant positive correlation with maturation date both in *C. macrocarpa* ($r_s = 0.530$, $P = 0.001$, n = 35 individuals, Spearman rank correlation) and *W. radicans* ($r_s = 0.504$, $P = 0.007$, n = 27 individuals).

Discussion

Environmental variables.—As expected, we found a decrease in temperature with an increase in altitude. The average altitudinal temperature gradient (−0.78°C/100 m) at Serra da Tronqueira was higher than those of other similar latitude regions. For example, in the Iberian Peninsula the gradient is −0.59°C/100 m in the northwestern coast (Carballeira *et al.*, 1983) and in the central mountain range (Wilson *et al.*, 2005). We also found an altitudinal moisture gradient. As in other Azorean islands (Borges, 1999), humidity increased with increasing altitude. Canopy cover varied among the three study sites as a consequence of different forest managements. At low and mid altitudes, the cover of the laurel forests was 71% and 63%, respectively. These percentages are lower than laurel forests in the Canary islands, with >90% canopy cover (Delgado *et al.*, 2007). At the low altitude site, the forest was highly disturbed, with a young native tree canopy invaded by *P. undulatum* and *A. melanoxylon*. At mid altitude, the management has opened gaps in the tree canopy where exotic species (mainly *C. arborea*) have been removed (SPEA, 2007). At high altitude, there was a dense mature *C. japonica* plantation creating a canopy cover of 83%, the highest of all the sites.

Spore maturation and abortion.—We determined the timing of spore maturation on the basis of perispore formation and lipid-drops accumulation. The spores of *C. macrocarpa* and *W. radicans* matured by autumn (mean maturation date November 11 and December 11, respectively). In both species spores have a high lipid and caloric content (Arosa *et al.*, 2009), as in ferns in general (Hew and Wong, 1974), but both spore mother cells and spores lack chloroplasts. Consequently, spore production may be largely controlled by the accumulation of enough photosynthates, as suggested for fruit production (French, 1992). In flowering plants, fruiting peaks generally occur during periods of low photosynthetic activity or after periods of high rates of reserve accumulation (Jordano, 1992). In temperate regions, fruiting has a unimodal peak in late summer or autumn (Jordano, 1992). The studied species mature spores in these seasons, as do many other temperate ferns (e.g., Page, 1997; Sawamura *et al.*, 2009), suggesting that production of seeds and spores are governed by analogous selective pressures. Leaf expansion of *C. macrocarpa* and *W. radicans* ends in early summer (Quintanilla, 2002). Thus autumn spore maturation depends on photosynthate accumulation during summer and autumn.

Maturation date was neither affected by altitude nor by canopy cover for either species. This indicates that temperature, humidity and light variation along the altitudinal gradient did not constrain resource build-up for spore production. Körner and Diemer (1987) found that the optimum temperature for photosynthesis of lowland herbaceous flowering plants was ~23°C in a temperate climate. In our study, temperatures were frequently close to this optimum in the three altitudes, especially during the summer. Relative humidity was also high in the three altitudes during the summer and autumn months (means > 85%) and thus water availability must not be a limiting factor. The photosynthetic responses to light have been studied in very few fern species (Page, 2002). *Hymenophyllum tunbrigense* and *H. wilsonii*, filmy-ferns that grow on *C. macrocarpa* shoots in the study area, show photosynthetic saturation at low light levels (Proctor, 2003). A similar ecophysiological response in the study species would explain why maturation date was not affected by the differences in canopy cover among sites (maximum 83%, minimum 63%).

Spore abortion can indicate environmental stress during sporogenesis. However, both species had few aborted spores at all three altitudes (means ≤ 8%) suggesting that environmental conditions are optimal for their growth. Values were similar to those obtained from *Dryopteris* spp. (< 10%) in populations in northern Spain (Quintanilla and Escudero, 2006).

Spore release.—In both species there was a long period between spore maturation (autumn) and release (late winter). Since spore dissemination is the only function of the sporangia, we might expect its phenology to be influenced by selective pressures which would favor successful dispersal. Delayed release could be a strategy to avoid unfavorable winter conditions. However, mean temperatures during winter were 9°C to 12°C depending on altitude (Fig. 1A), which are suitable for spore germination of the studied species (Quintanilla *et al.*, 2000).

The long-term causes of the timing of spore maturation and release can also be biotic pressures such as seasonal presence of spore feeders. Some temperate ferns are attacked by a variety of spore-predator insects which occasionally cause severe spore reduction (Sawamura *et al.*, 2009, and references therein). We have not observed spore-feeding insects in the study species but consumption by the Azores bullfinch is significant (Ramos, 1995; Arosa *et al.*, in press). Given that Azores bullfinch consumes mature spores, we have studied its potential disperser role and the results will be reported elsewhere. In short, many droppings contained high amounts of viable (able to germinate) spores of *C. macrocarpa* and *W. radicans* and thus may provide a vehicle for dispersal. The pattern of autumn spore maturation and late winter release occurs throughout the range of both ferns (own observation), while the Azores bullfinch is present only in a small fraction (one island). Thus, the negative (predation) or positive (dispersal) interaction with the Azores bullfinch may not be important for determining the timing of spore dispersal.

Spore dispersal may be largely influenced by evolutionary constraints. Related plants share similar inherent design constraints which would limit

their potential evolutionary response to selection (Fenner, 1998). In ferns, both indusia and sporangia openings are passively caused by evaporative forcing. This must be an adaptation to favor long-distance wind dispersal of spores in warm dry days. *Culcita macrocarpa* and *W. radicans* released spores a month earlier at low altitude than at high altitude. This is due to the humidity and temperature gradients (see above) that reduce the evaporative forcing at high altitude (Körner, 2007). Delayed spore release may be merely due to the absence of dry weather conditions during most autumn and winter days. Release date was positively correlated with maturation date, i.e., individuals with earlier spore maturation showed earlier spore release, indicating that there is some interdependence between these events.

Implications for conservation of Azores bullfinch.—*Culcita macrocarpa* and *W. radicans* spores, together with seeds of the exotic *C. arborea*, are the main winter foods for the Azores bullfinch (Ramos, 1995; 1996a). The birds took the whole sorus, rejecting the indusium. After spore release, sori are not consumed since empty sporangia have negligible nutritional value. Food supply is at its lowest at the end of winter and the mortality of first-year birds appeared greater in this period (Ramos, 1995, 1996b). A gradual release of spores along the altitudinal gradient is important for the maintenance of a stock of spores throughout the winter. We can envisage that the Azores bullfinch distribution should be progressively pushed up to higher altitudes along the winter season following spore availability. For effective conservation of Azores bullfinches, the populations of *C. marcrocarpa* and *W. radicans* must be increased along a wide altitudinal range. This is significant in terms of habitat restoration for the Azores bullfinch because present management actions aim to control the expansion of *C. arborea* (Ceia, 2008). This invasive tree, although important in the winter diet, has a negative effect in spring because it outcompetes the native *I. perado* and *P. lusitanica* (the flower buds of both species are the main early spring foods for the Azores bullfinch; Ramos, 1996b).

Conclusions.—*Culcita macrocarpa* and *W. radicans* have similar timing of spore maturation and release. Maturation is completed in autumn and is not affected by altitude nor by canopy cover. Spore production may be largely controlled by the previous accumulation of photosynthates. Spores of both species are not released until late winter due to a requirement for dry weather conditions. Dispersal occurs earlier at lower altitude, as a consequence of higher temperature and lower humidity. The present study is descriptive and thus cannot accurately establish cause-effect relationships. Transplant experiments moving individuals from one habitat to another or experiments manipulating the physical environment experienced by individual ferns will clarify the relative importance of environmental and genetic factors on spore phenology traits.

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Nutrient Levels Do Not Affect Male Gametophyte Induction by Antheridiogen in *Ceratopteris richardii*

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Abstract.—In the homosporous fern *Ceratopteris richardii*, sex is not determined chromosomally. Rather, hermaphroditic gametophytes produce a hormone called antheridiogen, which induces maleness in undifferentiated gametophytes. The percentage of males increases with increasing density of gametophytes, presumably due to the cumulative effect of antheridiogen from multiple hermaphrodites.

Some have argued that antheridiogen lessens competition between gametophytes. Such competition is expected to be most intense between hermaphrodites given that they support zygote, embryo, and sporophyte growth. Therefore, it is predicted that at lower nutrient levels, the effect of antheridiogen in inducing male gametophytes is greater than at higher nutrient levels.

To test this hypothesis, *C. richardii* spores were sown over a range of densities (0.52/cm^2 to 5.2/cm^2) in four nutrient-level treatments (100, 50, 25, 12.5 percent of full-strength nutrient agar). Gametophytes were grown for four weeks at 28 degrees Celsius with a photoperiod of 14 L: 10 D. An ANCOVA found an overall positive relationship between gametophyte density and percentage of male gametophytes. However, the relationship between gametophyte density and percentage of male gametophytes did not differ among nutrient levels. Nutrient levels had no effect on the rate of male induction by antheridiogen. A post-hoc power analysis showed that the experimental power was 97%.

Key Words.—*Ceratopteris*, sex determination, gametophyte, antheridiogen

The gender of sexually dimorphic plant species may be determined genetically, environmentally, or by a combination of the two (Lloyd and Bawa, 1984; Meagher, 1988). If sex in a species is determined by environmental factors, males should be more common in low-resource environments (Schlessman, 1988). In flowering plants, this occurrence has been explained by a higher cost of producing ovules, seeds, and fruits versus producing pollen (Lovett Doust and Harper, 1980; Lovett Doust and Lovett Doust, 1983). A similar tendency is seen in seedless vascular plants, in which the female/hermaphroditic gametophytes endure a higher reproductive cost because they are responsible for producing the egg, the zygote, and the embryo, as well as supporting the developing sporophyte (Sakamaki and Ino, 1999). Therefore, if sex determination in seedless vascular plants is analogous to flowering plants, then resource availability in the environment may be expected to have a direct effect on sex determination of gametophytes (Sakamaki and Ino, 1999).

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Specifically, males should be more common in environments with low resource levels.

Sex determination in the homosporous fern *Ceratopteris richardii* Brongn. is plastic. Spores develop into gametophytes that are either hermaphroditic, containing both archegonia and antheridia, or exclusively male, containing only antheridia (Banks, 1997). Developing and mature hermaphrodites produce a gibberellin-like hormone (Warne and Hickok, 1989) called antheridiogen (A_{CE} in *C. richardii*; Banks, 1997). A_{CE} induces maleness in developing gametophytes (Banks, 1997), provided that exposure occurs between the third and the sixth day after spore inoculation (Banks *et al.*, 1993; Eberle *et al.*, 1995). In the absence of A_{CE}, spores develop into hermaphrodites (Banks, 1997). This model of hormonally determined sex is not exclusive to *C. richardii* but is fairly common to many homosporous ferns (Haig and Westoby, 1988).

Multiple experiments have demonstrated that spore density has an effect on sex determination in *C. richardii* (Warne and Lloyd, 1987; Hickok *et al.*, 1995; Spiro and Knisely, 2008), as well as in another homosporous fern *Osmunda cinnamomea* L. (Huang *et al.*, 2004). More specifically, the sex ratio is skewed toward males at high gametophyte densities. One explanation for this observation is that at higher densities, undifferentiated gametophytes are clustered closer together within the neighborhood of multiple hermaphrodites and are thus exposed to the cumulative effects of A_{CE}. This hypothesis has indirect, empirical support in that experimentally elevating exogenous antheridiogen leads to male-skewed sex ratios in *C. richardii* (Warne and Hickok, 1991) and other species of homosporous ferns (Cousens and Horner, 1970; Stevens and Werth, 1999; Huang *et al.*, 2004). However, there is a maximum concentration of antheridiogen above which increasing the amount of the pheromone has no additional effect on sex ratios (Cousens and Horner, 1970; Stevens and Werth, 1999; Huang *et al.*, 2004).

An alternative hypothesis for the skewed sex ratios with increasing density relates to the resource cost of being male versus hermaphroditic. If the sex of the gametophytes is based on the resource state of the environment, or if the resource state alters the effects of exogenous A_{CE}, then the same pattern of gametophyte density and sex ratios would be expected: a higher ratio of males in low nutrient level culture and a lower ratio of males at higher nutrient levels for similar gametophyte densities. In high density cultures, resource levels per gametophyte are expected to be lower, while in low density cultures, resource levels per gametophyte are expected to be higher. The objective of this experiment is to determine whether the proportion of hermaphrodites in a culture of a given density may be altered by the level of nutrients.

Materials and Methods

Ceratopteris richardii Petri dish cultures were established on nutrient agar following Hickok and Warne (2004) using wild type *C. richardii* spores and powdered media obtained from Carolina Biological Supply Company.

Four experimental treatments were established using serial dilutions; 100% nutrient level, 50% nutrient level, 25% nutrient level, and 12.5% nutrient level. These nutrient levels are relative to the maximum level found in the powdered media. For a detailed list of nutrient components in media see Hickok and Warne (2004). Spores were sown on 35mm × 10mm Petri dishes containing the four nutrient treatments. Spore densities ranged from five to 50 spores per Petri dish and increased at increments of five spores. This yielded an overall density of 0.52 spores/cm^2–5.2 spores/cm^2. Each of the four nutrient-level treatments contained 40 Petri dishes (four at each of the 10 spore densities) for a total of 160 dishes.

Spores were incubated at 29 ± 3°C under grow lights (24 W/m^2) for 25 days following a 14 hours day/10 hours night cycle. At that time, determination of the sex of a high proportion of gametophytes was possible. The number of hermaphrodites, males, ungerminated spores, and spores of indeterminate sex were recorded. Hermaphrodites contain archegonia, antheridia, and a notch meristem, giving them a mitten-shaped appearance, whereas males contain antheridia, and are essentially oval (Banks, 1997). The shape of the gametophyte was the primary characteristic used for identification. The percentage of hermaphrodites in each Petri dish was determined by dividing the total number of hermaphrodites by the sum of the males and the hermaphrodites.

To determine if nutrient levels affected the proportion of hermaphrodites, an Analysis of Covariance (ANCOVA) was performed using SYSTAT (Wilkins, 2002). This analysis determined 1) whether the density of gametophytes was related to the proportion of hermaphroditic gametophytes and 2) whether the different nutrient level treatments had an effect on this relationship. The ANCOVA model included nutrient level as a categorical variable and density of gametophytes as a covariate. After testing for the homogeneity of slopes (i.e., the nutrient level*density of gametophytes interaction), the mean squares and degrees of freedom were excluded from the analysis (pooled into the error term) if $p > 0.05$.

With the lack of significance for the ANCOVA, a Power Analysis using G*Power (Faul *et al.*, 1996) was performed to assess the overall power of the experiment, as well as the probability of making a type II error (β). A type II error is one in which no effect is detected when an effect really exists (Winer *et al.*, 1991). The effect size necessary for power calculations followed Winer *et al.* (1991).

Results

Severe agar desiccation in two Petri dishes made it impossible to determine the sex of the gametophytes. As a result, 158 Petri dishes were used in the statistical analysis.

Ninety-nine percent of the gametophytes were identified as either males or hermaphrodites. The slopes of the covariate interactions (nutrient level*den-

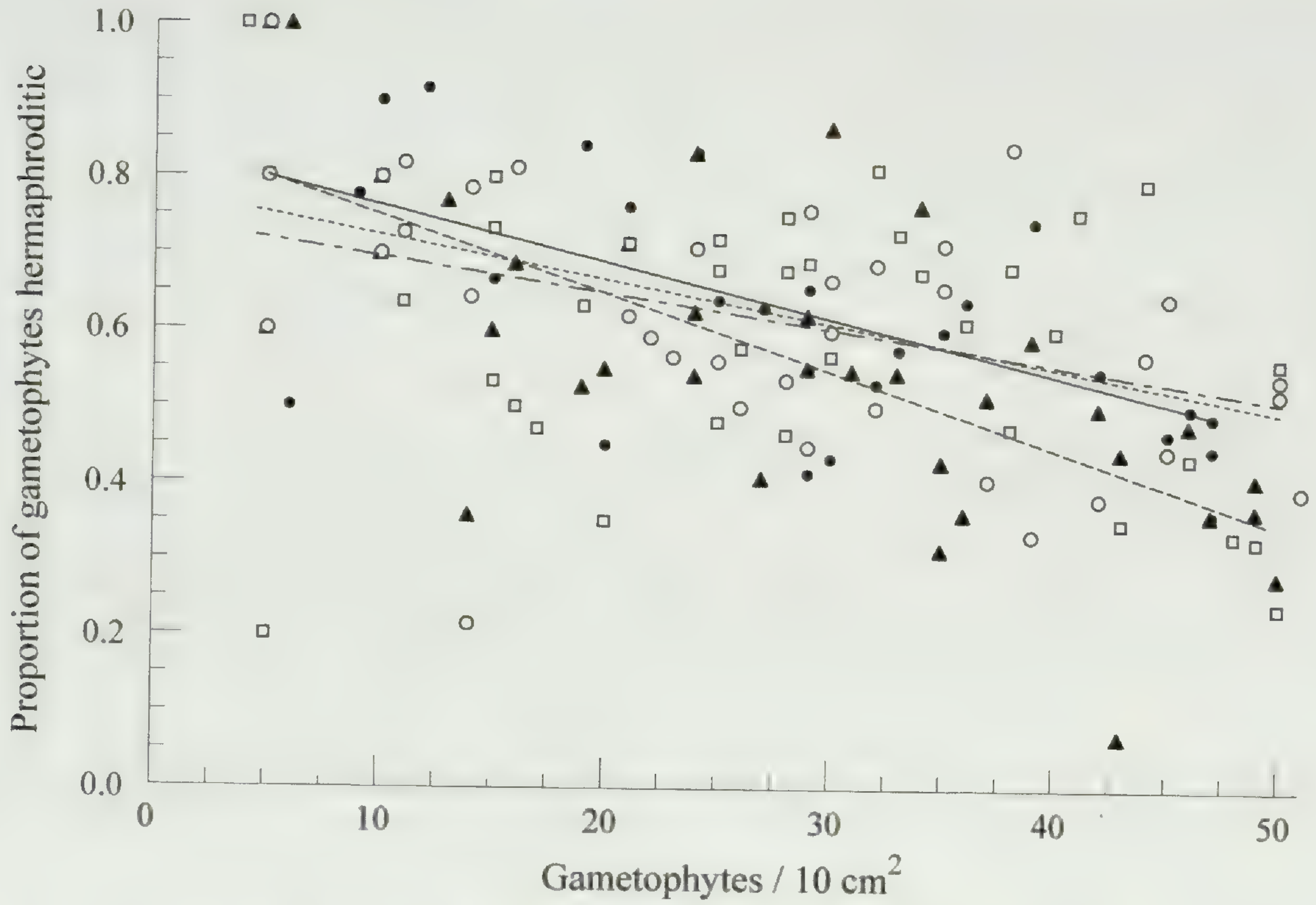

FIG. 1. Relationship between gametophyte density and the proportion of gametophytes hermaphroditic for each of the four nutrient treatments. Closed circle, solid line = 100% nutrient level; open square, dotted line = 50% nutrient level; open circle, dot-dashed line = 25% nutrient level; closed triangle, dashed line = 12.5% nutrient level.

sity of gametophytes) were homogeneous ($F_{3,150} = 1.932$; $p = 0.127$). Therefore, the final analysis did not include this interaction term.

There was an overall relationship between the density of gametophytes and the proportion of hermaphroditic gametophytes (Figure 1; $p < 0.001$, Adjusted $R^2 = 0.353$), in which the proportion of hermaphrodites declined with increasing gametophyte density. There was no effect of nutrient level on this relationship ($F_{3,150} = 1.113$, $p = 0.346$).

The statistical power to assess whether nutrient levels affected the relationship between the density of gametophytes and the proportion of hermaphroditic gametophytes required the calculation of an effect size. Effect size was determined to be 0.357 following Winer (1991). The probability of making a type II error, β, was determined to be less than 3%, making the power of this test greater than 97%.

DISCUSSION

Similar to previous studies of *Ceratopteris richardii* (Warne and Lloyd, 1987; Hickok *et al.*, 1995; Spiro and Knisely, 2008), this experiment demonstrated a negative relationship between gametophyte density and the proportion of

hermaphrodites that developed. Though this relationship was highly significant ($p < 0.001$), the adjusted R^2 was 0.353, indicating that only 35.3% of the variation in the proportion of hermaphrodites is explained by the variation in gametophyte density. The large amount of unexplained variation (64.7%) indicates that factors other than the presence or absence of A_{CE} likely influence sex determination in this species. Indeed, hermaphrodites have been shown to develop from young *C. richardii* gametophytes even in the presence of substantial antheridiogen (Warne and Hickok, 1991) or hermaphrodites (Sayers and Hamilton, 1995).

Other factors have been shown to override the effect of A_{CE} in *C. richardii*. Light quality, biased toward red light, suppresses male development in favor of hermaphroditic development (Kamachi *et al.*, 2007). Smaller spores tend to germinate later than larger spores, with the gametophytes of smaller spores tending to grow more slowly and to develop into males (Sayers and Hamilton, 1995).

While nutrients have been shown to affect sex ratios in at least one other fern, *Dryopteris filix-mas* (L.) Schott. (Korpelainen, 1994), no such effect was shown here for *C. richardii*. The nutrient levels used in this experiment did not alter the effectiveness of A_{CE}. Similar proportions of hermaphrodites were observed in cultures with similar gametophyte densities regardless of nutrient levels. The experimental power of this experiment was high (> 0.97) and therefore the probability of incorrectly concluding that nutrients have no effect on the proportion of hermaphrodites at similar densities is quite low (< 0.03).

The possibility that nutrient levels used in this experiment were not limiting cannot be discounted, although the lowest nutrient level used was 12.5% of the maximum. It is also possible that even though nutrient levels are not important in determining gametophyte gender, they are important to gender allocation within gametophytes. The relative number of antheridia and archegonia in hermaphrodites, or the number of antheridia present in the male may change in response to nutrient levels in the environment. The period of susceptibility to A_{CE} for undifferentiated gametophytes is between 3 and 6 days (Banks *et al.*, 1993). It is possible that at this young age, the nutrient quality of the environment is not discernable by the gametophyte. Therefore, other indicators of environmental quality, such as light, may be more likely to factor into sex determination.

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Transplanting Tree Ferns to Promote Their Conservation in Mexico

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Abstract.—Adult tree ferns of the genera *Cyathea* and *Alsophila* are frequently harvested from tropical forest remnants near the city of Cuetzalan (Puebla, Mexico). Local artisans use the adventitious roots that surround tree fern stems as substrate to make handicrafts. In this region, tree ferns regenerate abundantly in disturbed areas such as roadsides, in which they suffer high mortality due to weeding and other road maintenance activities. Transplantation of young tree ferns from these areas to safe sites could contribute to the *ex situ* conservation of the species. The sale of transplanted tree ferns could also provide local families with an additional source of income. We identified and estimated the abundance of all tree fern species that occurred along roadsides in this region. We evaluated the viability of transplanting young tree ferns of *Cyathea divergens* and *Alsophila firma* to different conditions of light availability. While only 30% of the individuals naturally growing along roadsides survived for 1 year, *C. divergens* transplants experienced 73.3 and 86.7% survival and *A. firma* transplants experienced 93.3 and 40% survival when planted in safe sites under open canopy and in 50% shade, respectively. Transplants of *C. divergens* produced more fronds and grew faster in height than transplants of *A. firma*. Individuals of both species transplanted to 50% shade produced more fronds and grew faster than conspecifics transplanted to open canopy areas. Transplantation proved to be a low time- and cost-demanding strategy to promote conservation of native tree fern populations while providing local people with a potentially profitable alternative to replace handicraft production.

Key Words.—*Cyathea*, *Alsophila firma*, management, Mexico, transplantation, tree fern, tropical montane forest

Disregarding law prohibitions, artisans in the region of Cuetzalan, Mexico harvest the stems of adult tree ferns of at least two species, *Cyathea divergens* var. *tuerckheimii* R.M. Tryon, and *C. fulva* M. Martens et Galeotti, to produce handicrafts (Eleutério and Pérez-Salicrup, 2006). Both species are listed in the Mexican law as threatened by land-use and land-cover changes (SEMARNAT, 2000).

Adult tree ferns are mostly harvested from natural populations that occur in remnants of tropical montane forests. These forests are among the most

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endangered ecosystems in Mexico (Diario Oficial de la Federación, 2001; Luna *et al.*, 2001). As a result, harvest contributes to increased mortality rates and jeopardizes the future regeneration of native tree ferns in forests that are already vulnerable (Eleutério and Pérez-Salicrup, 2006).

Although tree fern species are often restricted to shaded and high moisture sites, at least three *Cyathea* and one *Alsophila* species commonly establish and grow in ruderal habitats, such as roadsides, near Cuetzalan (pers. obs.). Therefore, any management policy aiming to preserve tree fern species in this region must consider their occurrence in different habitats (Werth and Cousens, 1990), from forest remnants to disturbed areas. However, no study has yet documented the establishment requirements of tree fern species in this region. Moreover, few studies have looked at management requirements for conserving the 13 *Cyathea* spp. that are currently considered endangered by the Mexican law (Diario Oficial de la Federación, 2001; Bernabe *et al.*, 1999).

Our study focused on providing basic information to allow the *ex situ* conservation of endangered species of the family Cyatheaceae. Tree ferns are usually propagated *ex situ* through spores, vegetative tissues (e.g., Finnie and Staden, 1987; Suzuki *et al.* 2005), and occasionally, by planting fronds (e.g., *Cibotium splendens* (Gaudich.) Krajina ex Skottsb.; Hensley, 1997) or the apical meristems of harvested adults (e.g., *Dicksonia antarctica* Labill.; Forestry Commission, 2001). Although not evaluated for the tree fern species that occur in the region of Cuetzalan, transplantation of seedlings and young plants is commonly performed to promote *ex situ* conservation and *in situ* enrichment planting of endangered species (Primack, 2002). Transplants are more likely to successfully establish and grow when environmental conditions of the sites where they naturally occur are similar to the ones of the areas they are relocated to (Jones and Hayes, 1999; Montalvo and Ellstrand, 2001).

For the species of Cyatheaceae native to the region of Cuetzalan, transplantation from roadsides to safe sites could be an important strategy for *ex situ* conservation. Along these roadsides, young tree fern sporophytes (≤ 50 cm tall) normally experience high mortality rates due to annual cutting of herbaceous vegetation for road maintenance (pers. obs.). A bank of sporophytes could be created by transplanting them from exposed to protected areas under adequate environmental conditions. If tree fern transplantation proves successful, cost and time associated with spore or gametophyte germination could be avoided. The sale of young tree ferns for horticultural purposes could potentially substitute for income obtained by selling handicrafts fashioned from tree fern adventitious roots, and consequently contribute to their *in situ* conservation.

To evaluate the feasibility of using transplants of *Cyathea* and *Alsophila* spp., extracted from roadsides for *ex situ* conservation, we first documented the tree fern species that naturally established along the margins of a major road used to access the city of Cuetzalan. We then transplanted young tree ferns to sites subjected to different light conditions to compare their survival and growth rates among species and treatments. Based on these data, we provide basic guidelines for tree fern conservation in the study region.

TABLE 1. Maximum stem height (m) of adults and altitudinal ranges (meters above sea level) in which the studied species of tree ferns typically grow.

Species	Adult maximum stem height (m)	Habitat Altitudinal Range (m.a.s.l.)
Cyathea divergens var. *tuerckheimii*	12	450–2400
Cyathea fulva	12	800–2700
Alsophila firma	10.5	750–2000
Cyathea costaricensis	8	250–750

METHODS

Study site.—This study was conducted in the vicinities of the city of Cuetzalan (20° 01′ 33″ N–97° 31′ 37″ W), in the northern region of the state of Puebla, central-eastern Mexico. Study areas were located at elevations ranging from 500 to 1470 meters above sea level (m.a.s.l.). Annual precipitation averages 4141 mm, with all months receiving > 100 mm of rainfall. Mean annual temperature is 19.4°C, ranging from 14.3°C in January, to 22.9°C in June (Instituto Mexicano de Tecnología del Água, 2000). The landscape is dominated by shade coffee plantations with diversified overstory tree canopies, and tropical montane forest remnants.

Study species.—Three species of the genus *Cyathea* and one species of the genus *Alsophila* were found along roadsides in a 670–1420 m altitudinal range: *C. divergens* var. *tuerckheimii*, *C. fulva*, *C. costaricencis* (Mett. ex Kuhn) Domin. (Mickel and Beitel, 1988), and *Alsophila firma* (Baker) D.S. Conant (Mickel and Smith, 2004). All species are protected by Mexican law (Diario Oficial de la Federación, 2001).

Cyathea spp. typically have trunks that range from approximately 10 cm diameter at breast height (DBH) to approximately 130 cm with the mantle of adventitious roots. Stems and stipes are scaly, and stipes may present spines (Mickel and Beitel, 1998). Adult stems are occasionally bent due to mechanical damage and to the posterior recovery of vertical growth (Seiler, 1981). Adults of both *C. divergens* var. *tuerckheimii* and *C. fulva* may present 12 m tall stems and grow in sites between 450–2400 and 800–2700 m.a.s.l., respectively. *Cyathea costaricencis* may grow to 8 m tall in relatively drier environments usually located between 250 and 750 m.a.s.l. (Table 1; Mickel and Beitel, 1998).

Species of the genus *Alsophila* also present scaled stems and stipes, but petiole scales have characteristic apical setae (Korall *et al.* 2007). Most species grow between 1000 and 2000 m.a.s.l. of elevation, rarely occurring at altitudes below 250 m.a.s.l. Adults of *A. firma* grow up to 10.5 m tall, and are typically encountered between 750 and 2000 m.a.s.l. (Table 1). Stems may branch by adventitious buds (Mickel and Smith, 2004; Tryon and Tryon, 1982).

Abundance of tree ferns along roadsides.—We counted, identified and measured the height of all tree ferns taller than 0.5 m growing within distances ≤ 2 m from the pavement along 16 km of the main highway to access the city of

Cuetzalan. This highway connects the region to the state's capital city, Puebla. We assigned each identified tree fern to the following five height categories: 0.5–1.0, 1.1–2.0, 2.1–3.0, 3.1–4.0, and > 4.0 m. In addition, we divided the tree ferns encountered along the roadsides into three altitudinal ranges: 670–920 m.a.s.l., 921–1170 m.a.s.l., and 1171–1420 m.a.s.l. We only sampled tree ferns above 0.5 m in height because we were interested in quantifying the abundance of individuals that had successfully established along roadsides.

Experimental transplants.—In October 2003, we collected tree ferns of the two species that presented a high number of individuals with a stem height between 10 and 50 cm, encountered within a 700–950 m.a.s.l. altitudinal range. Thirty *C. divergens* and 30 *A. firma* plants ranging 17–50 cm tall were excavated with spades from roadsides. Tree ferns were extracted with their entire root systems and approximately 1000 cm^3 of local soil. Plants within this altitudinal range were selected to minimize environmental heterogeneity between the site they were extracted from and the ones they were transplanted to, which were located at 700 m.a.s.l.

Fifteen transplants of each species were planted into a 50% shade greenhouse, and fifteen were planted in an open (full sun exposure) garden. Plants were transplanted within two hours into holes 20–30 cm diameter and 30 cm depth. Holes were filled with local soil mixed with organic compost. We cut all fronds with fully expanded pinnae to minimize transpiration. We marked all fiddleheads (i.e., emerging leaves) at the beginning of the experiment and in subsequent censuses. To evaluate growth rates we measured stem height (to the nearest 0.5 mm), from the base of the newest crosier to the soil surface, monitored frond production every 2.5 months for 1 yr (from October 2003 to November 2004), and reported mean values ± SE for the study period. Fronds with ≥ 10% green tissue were considered alive (*sensu* Durand and Goldstein, 2001).

To investigate the mortality of tree ferns along roadsides, in March 2003 we randomly selected 60 tree ferns < 30 cm tall of each *C. divergens* and *C. fulva*, the two species that presented a higher number of individuals in this size category, growing in altitudes ranging from 900 and 1200 m.a.s.l. This altitudinal range was selected to include the extension of roadsides that would be weeded during the period we performed our study. To verify the effect of the transplantation procedure, we used a spade to extract half of the plants of each species with their entire root systems. We subsequently replanted each plant into the same spot they had been extracted from (henceforth called transplanted control individuals). We measured stem heights before transplanting, and cut all mature fronds with fully expanded pinnae to reduce water loss. We monitored plant survival for both transplanted control individuals and non-transplanted individuals, every 3 mo from March 2003–April 2004.

We used failure time analyses to compare survival rates between species and between treatments in both experiments (Fox, 2001). We performed Cox proportional hazard model and log-rank tests (Pyke and Thompson, 1986) with SPlus 6.0 (Insightful Corp. Seattle, USA). We used linear and quadratic regressions between the initial and final stem heights to determine the model

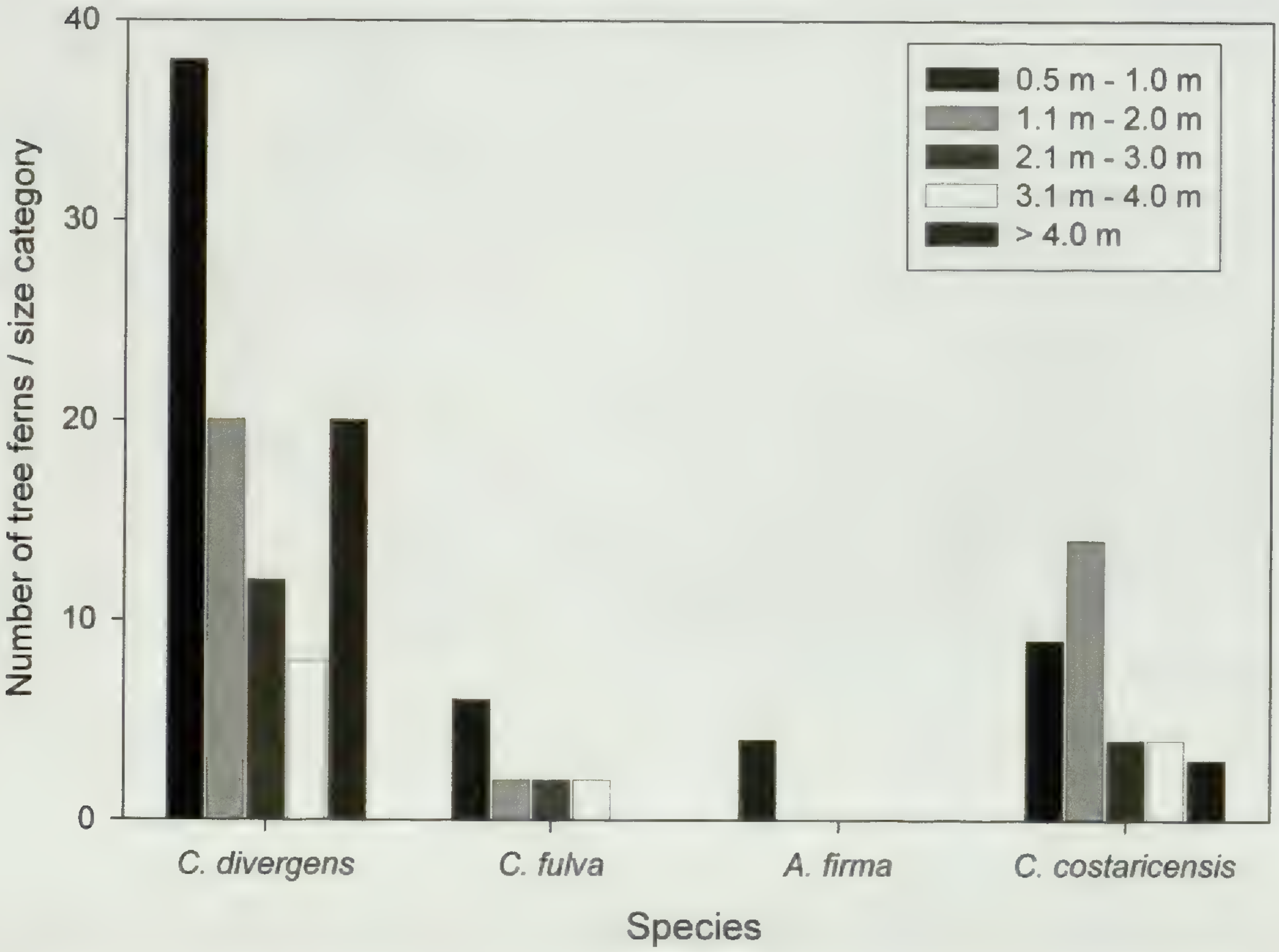

FIG. 1. Number of tree ferns in several size categories found for the three species of *Cyathea* and one species of *Alsophila* encountered along the roadside near the city of Cuetzalan del Progreso.

that best fit the patterns of stem growth (Sokal and Rohlf, 1995). Initial and final stem heights were linearly related, and therefore we performed a Pearson correlation analysis to evaluate the relationship between initial stems height and total number of fronds produced. We compared growth and frond production between species and treatments using a two way ANOVA without replication.

RESULTS

Abundance of tree ferns along roadsides.—Cyathea divergens and *C. costaricensis* were the most abundant tree fern species sampled along roadsides, with 96 and 34 individuals, respectively. Both species were represented in all height categories (Fig. 1). Only twelve plants of *C. fulva* and four of *A. firma* > 50 cm were encountered within the sampled area. Few plants taller than 3 m of both species were recorded (Fig. 1). *Cyathea divergens* was abundant along the whole altitudinal range sampled, while all the 34 plants of *C. costaricensis* were sampled within altitudes between 670 and 920 m (Fig. 2). Less than 10 individuals of *C. fulva* and *A. firma* were sampled in the whole study area. *A. firma* was restricted to altitudes between 921–1170 m, while *C. fulva* was only recorded in the other two altitudinal ranges.

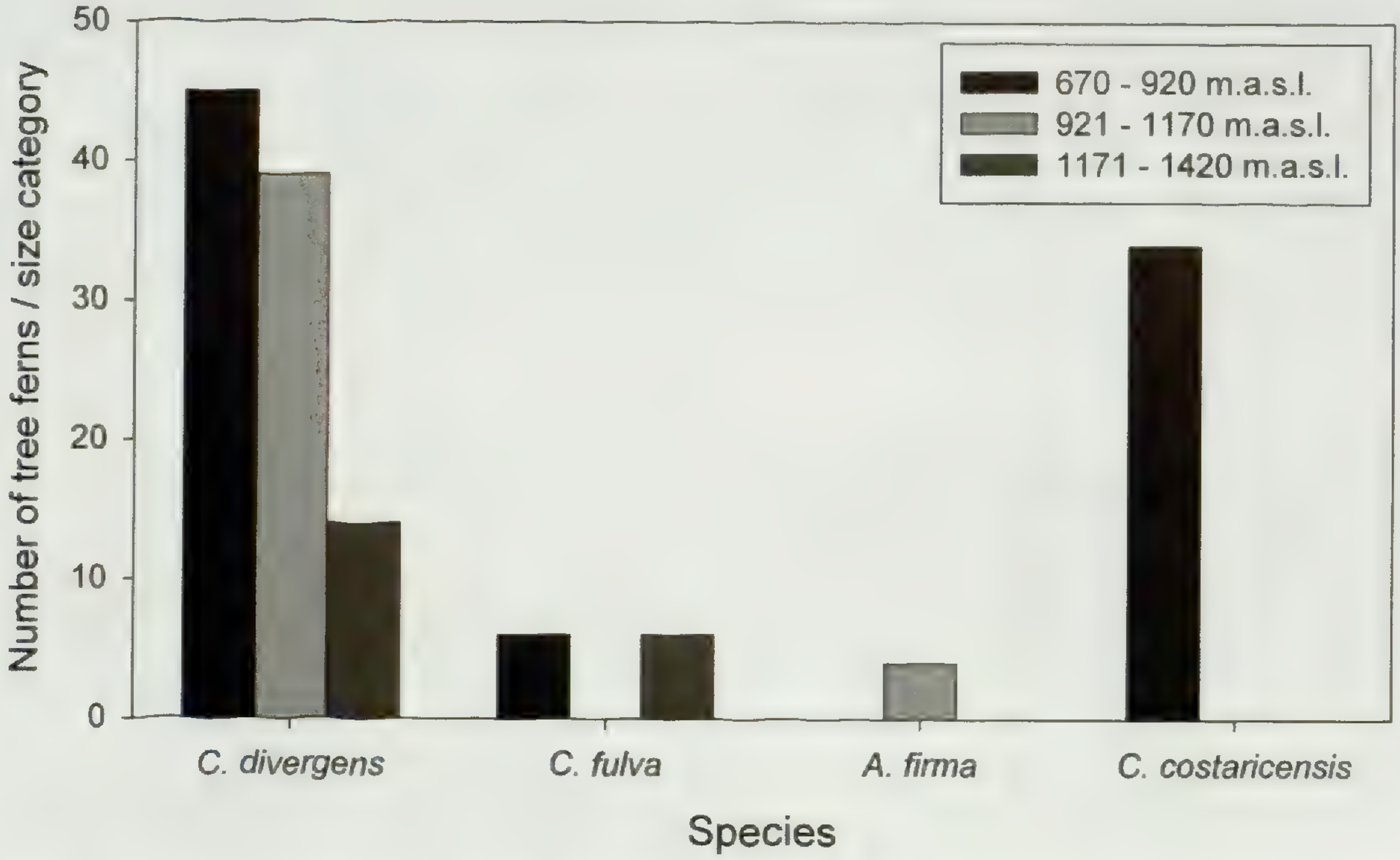

FIG. 2. Number of tree ferns of the four studied species by altitudinal range growing along the roadside.

Survival analyses.—While *C. divergens* experienced 73.3 and 86.7% survival, *A. firma* experienced 93.3 and 40% survival after 1 yr in open canopy and 50% shade, respectively. Survival curves did not differ between species (Fig. 3; log rank, $\chi^2 = 1.2$, df = 1, $P > 0.25$) or light treatments (Fig. 3; log rank, $\chi^2 = 3.2$, df = 1, $P > 0.05$). Survival rates were not affected by the initial height of stems ($z = -1.18$, $P > 0.2$), species ($z = 1.58$, $P > 0.10$), or light treatment ($z = -1.81$, $P > 0.05$) (likelihood ratio test = 6.58, df = 3, $P > 0.05$). Tree ferns of *C. divergens* transplanted to safe sites, whether open or shaded areas, survived more than those left on roadsides (control and transplant control; see Figs. 3 and 4). Less than 50% of the transplants of both *C. divergens* and *C. fulva* survived for more than 6 mo when left along the roadsides. After 1 yr from the beginning of the experiment, approximately 37% of the control individuals of *C. fulva* survived, while less than 10% of *C. divergens*, or of the plants assigned to the other treatments, survived (Fig. 4). In general, plants kept as controls had greater survival than the controls for the transplantation method.

Growth analyses.—Stem growth in height was between 4–5 mm/mo either in the sun or shade treatments and for both studied species in the first months after transplantation. Frond production was lower in the first three months than subsequent months (approximately 0.6 fronds/mo, in comparison to the 1.0–1.2 fronds/mo observed during the subsequent period). For these reasons, we decided to use data from the whole censused period to calculate total stem growth and frond production. Mean height growth rates for *C. divergens* were 8.0 ± 1.0 mm/mo in open canopy, and 13 ± 2.1 mm/mo in 50% shade. Mean

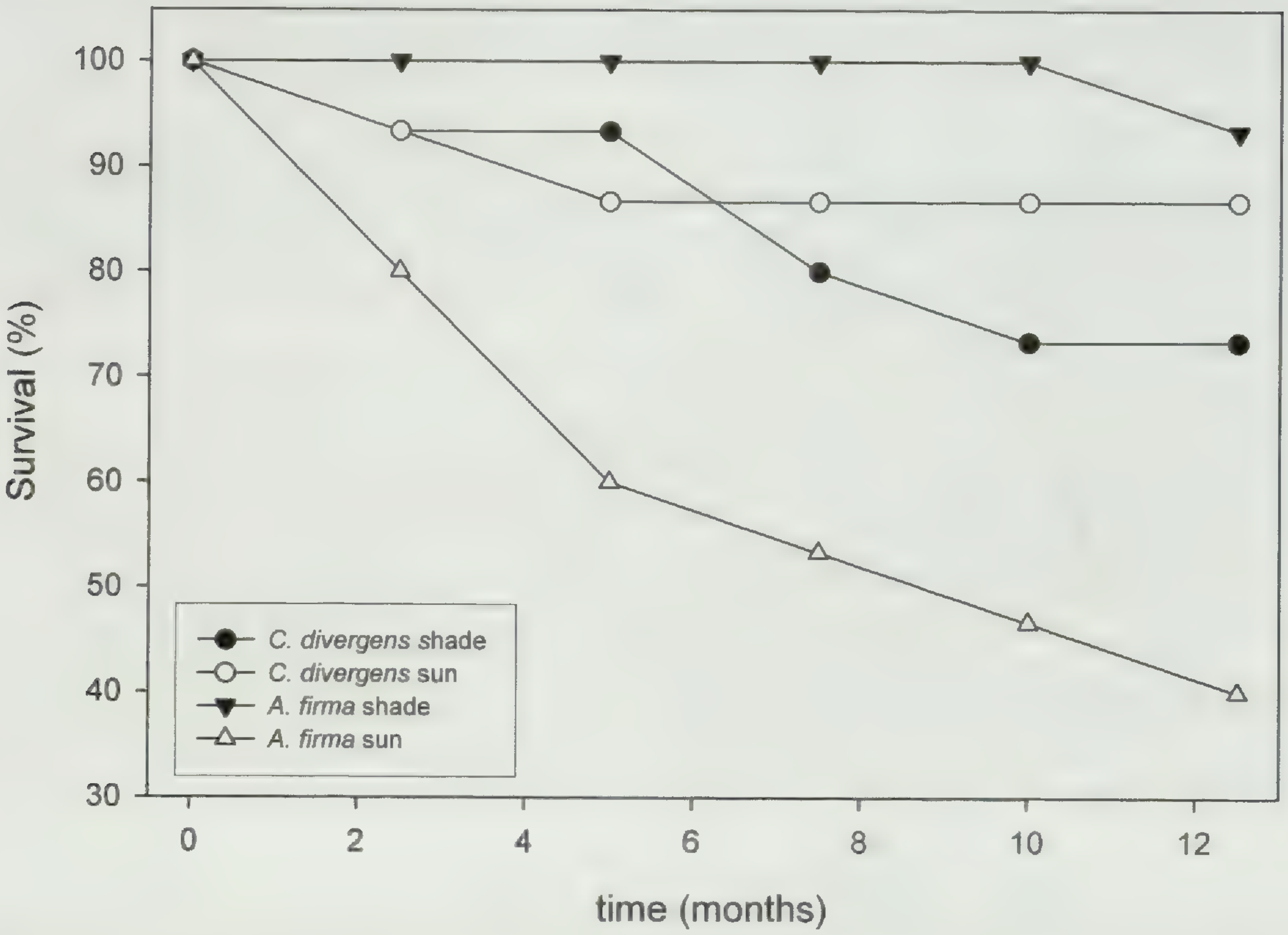

FIG. 3. Survival curves of transplanted *Cyathea divergens* and *Alsophila firma* individuals to a 50% shaded greenhouse and an open common garden near Cuetzalan del Progreso, Puebla.

height growth rates for *A. firma* were 2.0 ± 1.4 mm/mo in open canopy and 6.0 ± 0.8 mm/mo in 50% shade. Height growth rates were higher for *C. divergens* than for *A. firma* (Fig. 5(A); ANOVA, $F_{1,41} = 23.0$, $P < 0.001$), and were higher under shade than in sunny conditions (Fig. 5(A); ANOVA, $F_{1,41} = 9.0$, $P < 0.001$).

Frond production was not related to initial stem height (*C. divergens*: Pearson, $r < 0.65$, $P > 0.5$ in shade and $r < 0.5$, $P > 0.5$ in sunny conditions; *A. firma*: Pearson, $r < 0.6$, $P > 0.25$ in shade and $r = 0.295$, $P > 0.95$ in sunny conditions). Consequently, this variable was not considered as a covariate in the comparisons between species and treatments. Individuals of *C. divergens* produced 13.9 ± 0.85 and 15.5 ± 0.89 fronds/yr in open canopy and 50% shade, respectively, while *A. firma* individuals produced 6.8 ± 0.97 and 10.4 ± 0.53 fronds/yr in open canopy and 50% shade, respectively. *Cyathea divergens* produced more fronds per yr than *A. firma* (Fig. 5(B); ANOVA, $F_{1,35} = 47.5$, $P < 0.001$). Both species produced more fronds under 50% shade than in open canopy (Fig. 5(B); ANOVA, $F_{1,35} = 7.8$, $P < 0.001$).

DISCUSSION

Cyathea divergens was the most abundant tree fern species found along roadsides near Cuetzalan. This might result from a higher abundance of the

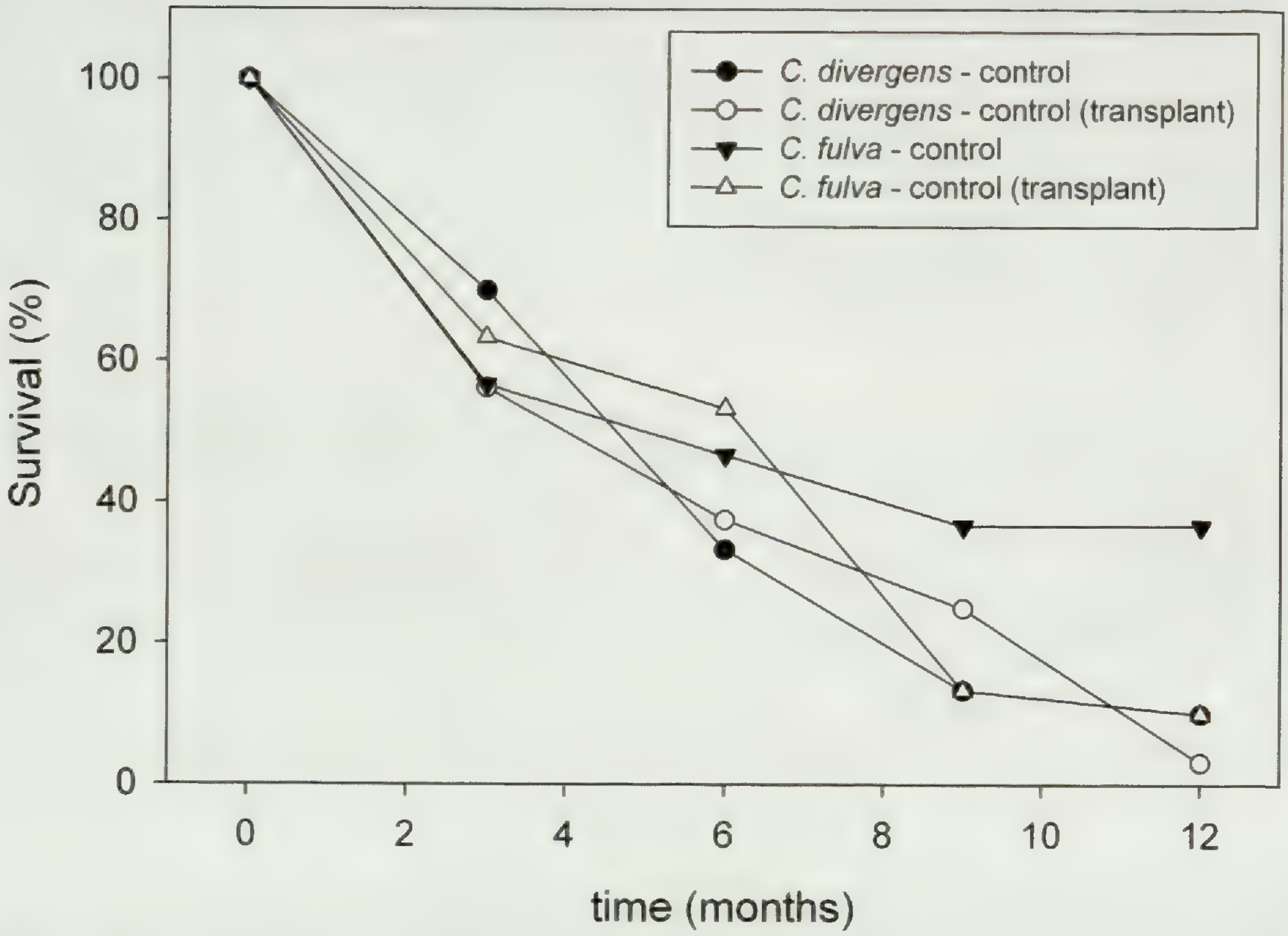

FIG. 4. Survival of transplanted *Cyathea* spp. under two experimental treatments in Cuetzalan del Progreso, Puebla. Treatments corresponded to: (1) control – individuals marked on roadsides; (2) transplanted control – individuals marked and transplanted to the roadsides areas from where they were extracted.

species in the region, together with an adaptation to environmental conditions in disturbed areas. In contrast, we encountered only young individuals (< 1.0 m in stem height) of *A. firma*, all located within an intermediate altitudinal range. Apparently, the conditions experienced in disturbed areas are not appropriate for the establishment and long term survival of this species. All studied species, except *C. divergens*, were unevenly distributed across altitudinal ranges. Air humidity and temperature, soil moisture content, and other environmental conditions that vary with elevation may restrict the habitat range of the less adaptable species. This distinct species' abundance and the variation in the number of tree ferns sampled in each altitudinal range have to be considered for selecting proper sites for extracting seedlings or transplanting them. In addition, species' abundances in natural populations and in a broader range of disturbed areas should be assessed in order to provide reliable management and conservation strategies for tree ferns in the area.

A higher number of transplants of *C. divergens* survived when planted in safe sites, whether they were located in sunny or shade conditions, compared to the controls left along roadsides (see Figs. 3 and 4). In such sites, tree ferns recurrently suffer damage due to weeding. Damage may have stressed young

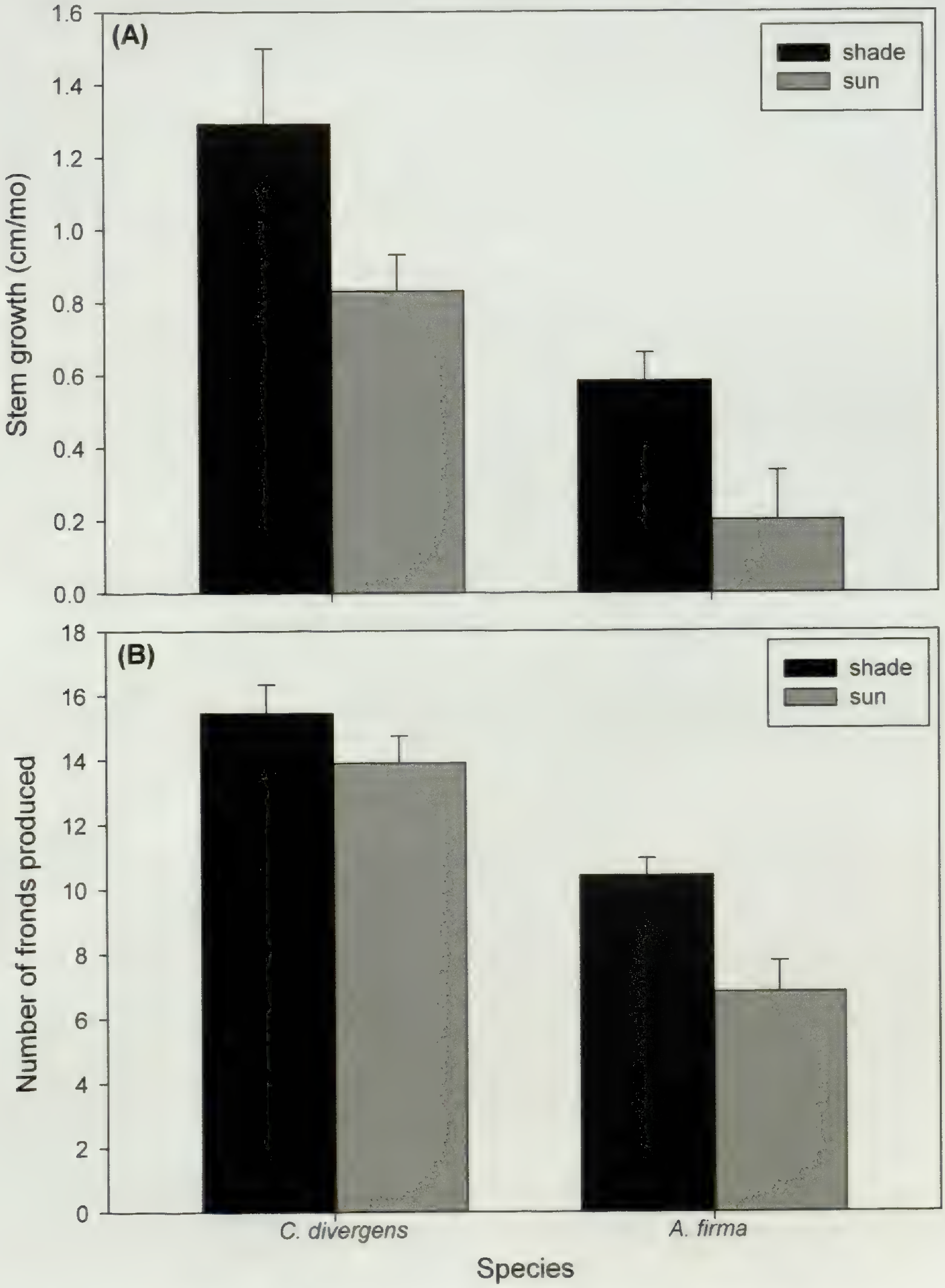

FIG. 5. (A) Height growth (± SE) for transplanted individuals of *Cyathea divergens* ($N = 11$ shaded, $N = 13$ in the open) and *Alsophila firma* ($N = 14$ shaded, $N = 6$ in the open) near Cuetzalan del Progreso, Puebla. (B) Production of new fronds (± SE) in transplanted individuals of *Cyathea divergens* ($N = 11$ shaded, $N = 10$ in the open) and *A. firma* ($N = 12$ shaded, $N = 5$ in the open) near Cuetzalan del Progreso, Puebla. All differences were statistically significant ($P < 0.05$).

tree ferns beyond their capability to recover, causing their death. Although not statistically significant, survival rates of *C. fulva* were higher in control than in control transplant treatments after 1 yr. Tree ferns of this species may not overcome the stress caused by transplantation. Further studies are necessary to assess the adequate conditions that would increase the survival of transplants of *C. fulva*. In general, survival rates were greater when tree ferns were transplanted to safe sites. However, because our experiments do not allow for comparisons among sites for species other than *C. divergens*, more solid information about the survival of transplants will depend on future experiments, in which each species is subjected to all treatments.

Survival rates were statistically similar for tree ferns relocated to safe sites under sunny or shade conditions. Bernabe *et al.* (1999) observed the same pattern for two of the three tree fern species they studied in tropical montane forests of Mexico. As suggested by these authors, tree fern species vary in their tolerance to different light conditions. In our study, both *C. divergens* and *A. firma* seemed to tolerate a wide range of light availability. However, the survival rate for *A. firma* planted in the shade was higher, although not statistically significant, than in the sun treatment. The ability to branch by adventitious buds may confer this species a higher tolerance to the stress caused by transplantation. For *A. firma*, shade conditions, in which water stress is diminished, may be ideal for transplantation, at least during establishment, until the transplants produce their first expanded leaves. Further differences in survival could have been observed if younger and, therefore, more vulnerable, transplants were used, and if we had a bigger sample size or our observations were prolonged for more than 1 yr.

Several studies on tree ferns have focused on understanding the plasticity of phenological responses of many species and the effects of such plasticity on population dynamics (e.g., Hunt *et al.*, 2002; Mehltreter and García-Franco, 2008). Adaptations to different light conditions have particularly been addressed for several species (Arens, 2001; Ash, 1987; Bernabe *et al.*, 1999; Seiler, 1981, 1984; Walker and Aplet, 1994). In sunny conditions, for example, several *Cyathea* species often grow faster in height, produce more fronds and start to reproduce earlier than in shady conditions (Arens, 2001; Ash, 1987; Bittner and Breckle, 1995; Poulsen and Nielsen, 1995). On the other hand, *Dicksonia antarctica* Labill seems to grow slower in sunny sites, when compared to less exposed and more humid sites, where they are less likely to experience water stress (Hunt *et al.*, 2002). Different light intensities can also cause changes in crown architecture of some *Cyathea* species (Arens and Sánchez-Baracaldo, 2000; Cox and Tomlinson, 1985; Tanner, 1983).

In our study, transplants of both *C. divergens* and *A. firma* placed in the shade grew faster in height and produced more fronds than those planted in sunny sites. Our data is in contrast to what has been observed for other tree fern species, which grow faster when exposed to conditions of higher light availability (see Arens, 2001; Arens and Sánchez-Baracaldo, 1998; Ash, 1987; Bittner and Breckle, 1995; Bernabe *et al.*, 1999; Seiler, 1981). These results may reflect a faster adaptation of transplants to shade conditions. When planted in

the shade, tree ferns would experience lower air temperature, higher air humidity and soil moisture availability. Under such conditions, water uptake would probably be adequate, and transpiration rates would not be elevated. Photosynthesis limitation by water availability would probably be lower when transplants are placed in the shade, in comparison to sunny sites, allowing tree ferns to grow faster and produce more fronds.

Transplantation is a low cost- and time-demanding activity that would adequately enhance both *in* and *ex situ* conservation of tree fern species in the region of Cuetzalan. Potentially, young tree ferns transplanted from sites in which they experience elevated mortality could be used as ornamental plants, promoting the *ex situ* conservation of native *Cyathea* spp. in the region. Our study suggests that transplants of *C. divergens* could be successfully used in gardening. Under adequate conditions, this species showed high survival and growth rates. More concrete conclusions should rely on complementary studies with a larger number of transplants by species, divided into replicate sites.

Additional support for the *ex situ* conservation of *C. divergens* comes from the fact that it is probably the most harvested species for handicraft production (Eleutério and Perez-Salicrup, 2006). Transplants could also be used for the conservation of rare species, such as *A. firma*, which is considered to be at risk of extinction (Diario Oficial de la Federación 2001).

Many local farmers, for example, have manifested interest in transplanting tree ferns to the understories of their shade coffee plantations. Given that our study shows that young tree ferns should be preferentially transplanted to sites where they are not exposed to direct sunlight, this use by local farmers may be successful. Moist and shaded conditions provided by the canopy of shade coffee plantations are probably adequate for a successful establishment and growth of transplants. Tree ferns could potentially survive and satisfactorily grow associated with this land use if they are protected from accidental damage during agricultural activities. If a few requirements are met, transplantation might also be the opportunity for local farmers to engage in the responsible management and trade of transplanted *Cyathea* spp. individuals.

The trade of more abundant and less endangered tree fern species would additionally require more than current market assessments and future market predictions. Detailed studies about the state of native tree fern populations occurring in forest remnants in the region are essential to provide policies that benefit *in situ* conservation and limit tree fern exploitation. In addition to limiting tree fern exploitation, the extraction of tree ferns from disturbed areas and native populations in forest remnants should be exclusive to local landowners, who depend on the exploitation of forest natural resources for their livelihoods (see Pérez-García and Rebollar-Dominguez, 2004).

Finally, our study emphasizes the importance of disturbed areas for the conservation of endangered species. These areas may constitute not only important sources of young tree ferns, but also seedlings of other species.

Therefore, the use of transplants for promoting conservation is worth testing for other species, sites, and environmental conditions.

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Mycorrhizal Associations in Ferns from Southern Ecuador

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ABSTRACT.—We conducted a survey on the mycorrhizal status of neotropical ferns, focusing on previously neglected taxa. These include the filmy ferns (Hymenophyllaceae), grammitid ferns (Polypodiaceae), and the genus *Elaphoglossum* (Dryopteridaceae). Samples were collected at different sites in southern Ecuador, Prov. Loja, Morona-Santiago, and Zamora-Chinchipe. Among the 85 investigated species (101 samples, 10 families), 19 were associated with arbuscular mycorrhizal fungi (AMF) and 36 were infected by dark septate endophytes (DSE), which are identified as ascomycetes and here considered as a kind of mycorrhiza similar to the ericoid type. The roots of 30 species (including all non-grammitid Polypodiaceae and half of the *Elaphoglossum* species) were free of evident fungal infection. AMF were frequent in terrestrial species (29.10% of species, or 48.49% of infected terrestrial samples). DSE prevailed in epiphytic species (58.62% of species, or 96.15% of infected epiphytic samples) and were also common in terrestrial samples of predominantly epiphytic species.

KEY WORDS.—Andes, arbuscular mycorrhizal fungi (AMF), ascomycetes, dark septate endophytes (DSE), grammitid ferns, Hymenophyllaceae, vesicular arbuscular mycorrhizae (VAM)

Mycorrhiza, the symbiosis between fungi and plant root, is known to enable plants to survive in the harshest environments by mediating nutrient and water fluxes (Allen *et al.*, 2003; Cairney and Meharg, 2003; Cooke and Lefor, 1998). Despite the evident advantage, there are conditions under which plants may dispense of a fungal partner and thrive, especially if they are growing on substrates with easy nutrient availability. Since most plant groups have a preference for one type of substrate, it does not surprise that mycorrhizae are

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unevenly distributed among the plant families (Newman and Reddell, 1987; Wang and Qui, 2006). Each new screening for fungal infections helps to understand the relationship between substrate type and mycorrhizae, especially if they include exceptions from the rule (e.g., Gemma *et al.*, 1992; Moteetee *et al.*, 1996).

Mycorrhization is common and diverse among landplants (Brundrett, 2002, 2004; Allen *et al.*, 2003) but only two types have been confirmed for ferns and lycophytes. The arbuscular mycorrhizal fungi (AMF) belong exclusively to the Glomeromycota (Schüßler *et al.*, 2001; Brundrett, 2004) and are the oldest form of the symbiosis (Pirozynski and Malloch, 1975; Blackwell, 2000; Brundrett, 2002). They are prevailing among ferns, lycophytes, and most other groups of vascular land plants (Brundrett, 2004). AMF are unable to grow without the association to a green plant (Brundrett, 2002), and are not easily dispersed from the soil to other habitats (Janos, 1993). The other group is the dark septate endophytes (DSE), which is a polyphyletic compound of several more derived fungal lineages. Contrary to the AMF, their spores get airborne more easily and are thus more readily available in the epiphytic habitat. The symbiotic character of DSE associations is still discussed controversially because the taxa involved are closely related to non-symbiotic endophytes, pathogens, and litter decomposers (Jumpponen and Trappe, 1998). However, most DSE found in ferns are apparently related to the ascomycetes (Schmid *et al.*, 1995) that form the well-studied Ericoid mycorrrhiza (Cairney and Meharg, 2003). Basidiomycetes (i.e., the known showy mushrooms) are commonly associated with northern temperate tree species and most orchids, including the epiphytic species (Brundrett 2004). Although they can also be found in liverworts (Kottke and Nebel, 2005), they are not confirmed as fungal partners of ferns and lycophytes (Kottke *et al.*, 2008).

Compared to the overwhelming diversity of green plants in the tropics, the studies on tropical mycorrhizae are relatively few (Wang and Qiu, 2006). One area worthy of such investigations is the Reserva Biológica San Francisco in southern Ecuador (Prov. Zamora-Chinchipe), where we conducted ecological studies on ferns and lycophytes (Gradstein *et al.*, 2007). The 1000 ha large reserve contains mature montane rain forest at 1800–3150 m and harbors 247 species of ferns (incl. horsetails; Smith *et al.*, 2006) and lycophytes (Lehnert *et al.*, 2007). The rugged topography of the area creates a mosaic of different substrate properties, with nutrient deficient soils on the ridges (Gradstein *et al.*, 2008) and slopes that receive a downhill flow of nutrients (Wilcke *et al.*, 2001). The divergent soil properties should also influence the mycorrhization of the plant species, given the fact that mycorrhizae enable plants to prosper in harsh nutrient deficient environments (Cairney and Meharg, 2003). Surprisingly, many usually epiphytic species in the area also colonize the ground on the ridges (Kessler and Lehnert, 2009), although epiphytic ferns are considered to be less dependent on mycorrhizae than terrestrial ones. Highly abundant groups with numerous epiphytic species in the area are the filmy ferns (Hymenophyllaceae), grammitid ferns (Polypodiaceae), and the genus *Elaphoglossum* (Dryopteridaceae).

Looking for a reference on the mycorrhizal status for these fern groups, we found that most available reports are for smaller regions outside of South America (e.g., Berch and Kendrick 1982; Cooper 1976; Gemma *et al.*, 1992; Iqbal *et al.*, 1981; Moteetee *et al.*, 1996; Nadarajah and Nawawi, 1993), and the few surveys cover only a fraction of the ferns and lycophytes worldwide (Boullard, 1958, 1979; Hepden, 1960; Newman and Reddell, 1987). No treatment for tropical Andean ferns was found; the few studies in South and Central America had either no overlap in the investigated species (Andrade *et al.*, 2000; Fernández 2005), or they had contradicting results for the same species (Lesica and Antibus, 1990; Schmid *et al.*, 1995). Compared to the general diversity, the number of investigated species from our three focal groups (filmy ferns, grammitid ferns, and the genus *Elaphoglossum*) is rather low. The present account aims to increase the investigated species number of these groups in order to have a more representative basis for future comparative studies.

Boullard (1958) included several Neotropical species in his survey but these were sampled either from herbarium specimens or from cultivated material. Drying reduces the ability of the hyphae to take up the dye, so that the mycorrhization of the plant may be rated too low or may go undetected. In cultivation, the kind or degree of mycorrhization may depend on the fertilization of the substrates (Entry *et al.*, 2002). Species that otherwise are mycorrhizal may completely dispense of the symbiosis in cultivation. Therefore, root samples are best taken directly from nature and preserved specifically for later dyeing. As far as we know, this is the first survey on mycorrhizae in tropical Andean ferns sampled *in situ*.

Materials and Methods

Root samples were collected at different sites in SE Ecuador: A) along the Gualaceo-Limon road (3100–3300 m, Prov. Azuay), B) the mountain pass El Tiro between the towns of Loja and Zamora (2600–2800 m, Prov. Loja/Zamora-Chinchipe), C) the area of Cerro Toledo, situated E of the town of Yantzatza (2900–3100 m, Prov. Loja), D) Reserva Biológica San Francisco (1800–2600 m, Prov. Zamora-Chinchipe), E) Reserva Cajanuma (2750 m, Prov. Loja), F) Reserva Tapichalaca (2450–2650 m, Prov. Zamora-Chinchipe), and G) the Campamento Indígena Shaimi on the shores of Río Nangaritza (900–1200 m, Prov. Zamora-Chinchipe). The study sites span an elevational gradient of 2400 m and range from lower montane forest to páramo vegetation. All sample areas face east and receive heavy precipitation all year round (Richter, 2003).

Sampling was focused on previously rarely investigated taxa. The substrates of the ferns were categorized as terrestrial, epiphytic, and saxicolous (= epilithic, rupicolous). Voucher specimens were deposited at Pontificia Universidad Católica del Ecuador, Quito (QCA). Duplicate collections of M. Lehnert were further distributed to Göttingen (GOET) and Berkeley (UC), and a set of specimens collected by L. Pazmiño is deposited at the herbarium of Universidad Técnica Particular de Loja (UTPL), Ecuador.

Sample plants were carefully removed and cleaned mechanically from the substrate, then rinsed with water to remove smaller litter parts and mineral compounds. At least 10 cm of roots from each specimen were preserved in 70% ethanol; of plants which we suspected to harbor DSE, additional 5–10 cm of the roots were preserved in 10% aqueous glutardialdehyde for transmission electron microscopy (TEM) preparation and stored at 8–10°C.

Preparation of the ethanolic samples for light microscopy followed Grace and Stribley (1991) and Haug *et al.* (2004). The samples were cleared in 10% KOH for ca. 24 h at 60°C; if the roots were still dark, the KOH was changed and the sample was kept at 60°C for another 12–24 h. Then the roots were rinsed twice with water and acidified with 1 N HCl. Staining was done with 0.05% methyl blue in lactic acid for at least 3 h. The stained roots were examined with a dissecting microscope at 30–60 ×; promising young roots were cut into portions, mounted on slides in lactic acid and examined at 100–400 ×. If mounted roots turned out to be insufficiently cleared, they were bleached with 3 % H_2O_2 for 2–5 min, rinsed with water and acidified with 1 N HCl. Then they were covered with same staining solution as before and heated over a small flame for 1–3 min. Excess staining solution was washed off with 90% lactic acid.

Preparation of the TEM samples followed Schmid *et al.* (1995). We opted for the fixation with 1% osmiumtetroxid for 1 h at 20°C, then 1% uranylacetate for 1 h at 20°C. Samples and slides are stored at the Georg-August-Universität Göttingen, Germany.

AMF were screened in the light microscope for presence. AMF are recognizable as relatively strong, aseptate hyphae with irregular diameter, forming terminal and lateral vesicles (Boullard, 1958). These infections were counted as real mycorrhizae if arbuscules were visible in the cortex (Gemma *et al.*, 2002).

Dark septate endopyhtes (DSE) were assigned to ascomycetes (Schmid *et al.*, 1995) if the characteristic Woronin bodies at the porate septa in the hyphae were visible in the TEM (Fig. 1D; Haug *et al.*, 2004). Fungal infection was considered as mycorrhiza if hyphal coils were developed in host cells that were still intact and showed some response to the infection, i.e., thickening of the cell walls where the hyphae penetrated the cell and thickening of host cell cytoplasma as indicator of increased cytological activity (Fig. 1C).

The frequency of infections in the roots was quantified under the light microscope, preferably on a single root with a minimum length of 10 cm measured from the root tips. In cases where the plants developed only considerably shorter roots, we combined several complete roots to reach the minimum length of 10 cm. The frequency of stained hyphae was categorized in three classes (Gemma *et al.*, 1992) to give an impression of the extent of the infection: Present in 1) <25%, 2) 25–75%, and 3) >75% of investigated root length. Presence of single hyphae or vesicles in the outer cortex as well as infection rates below 5% were considered as erroneous infections and not counted as mycorrhizal association. We did not distinguish between "obligately" and "facultatively mycorrhizal" because we usually sampled

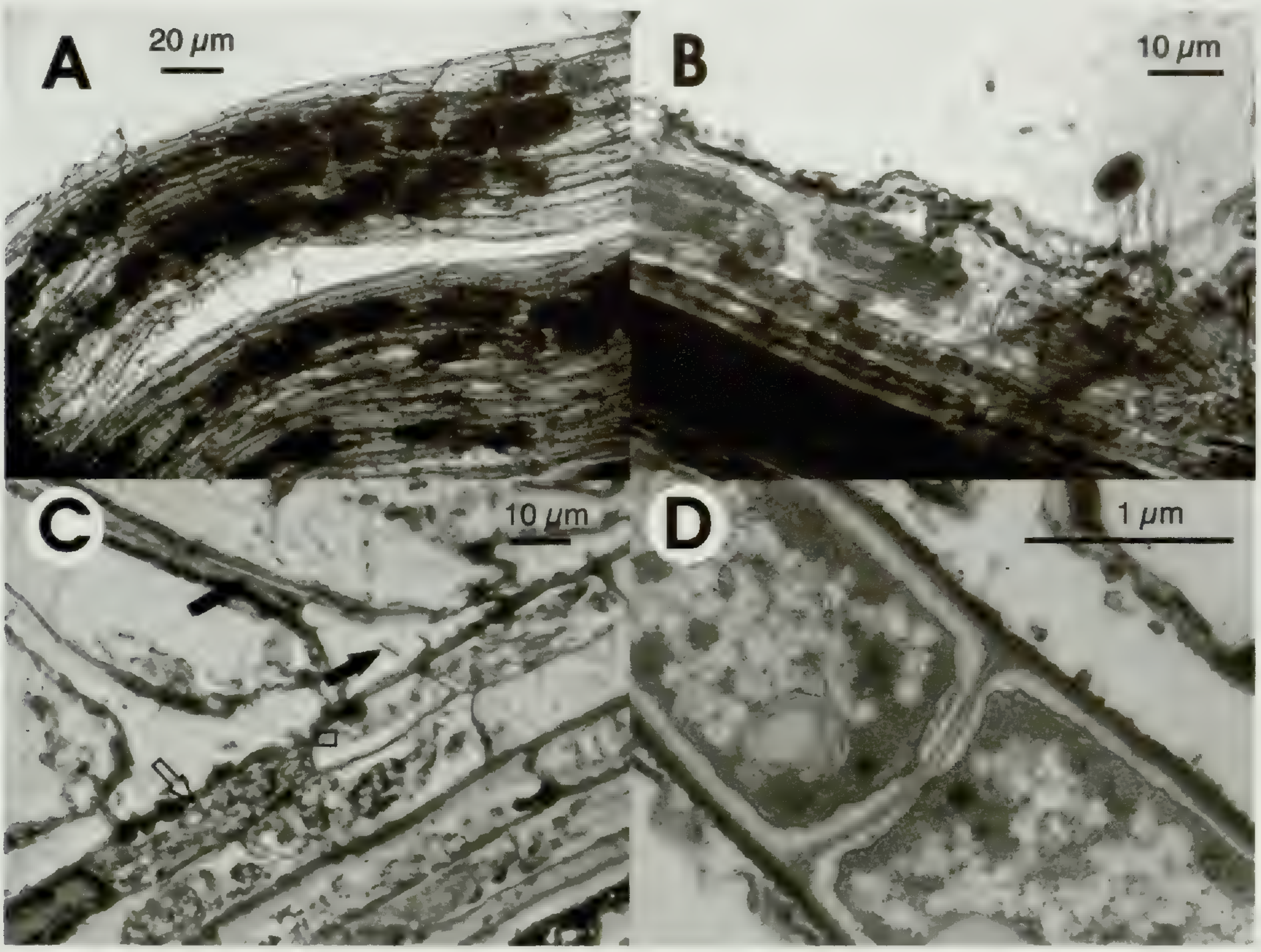

FIG. 1. Fungal infections in fern roots. A) AMF infection in young roots of *Loxsomopsis pearcei*; arbuscules fill out most of the cortical cells. Photograph M. Lehnert; B) DSE infection of root cortex in *Lellingeria major*, visible as external net and dense, internal coils of hyphae. Photograph M. Lehnert; C) Infection by ascomycete in *Melpomene firma*; the hyphae enter the root through the root hair tips (black arrows) and form dense hyphal coils in the outer cortex (outlined arrow). Photograph M. Lehnert; D) Infection by ascomycete in *Melpomene firma*; detail of septum with characteristic Woronin-bodies, visible as darker dot on each side of the septum. TEM-photograph I. Kottke.

only one specimen per species and habitat. Since degree and frequency of mycorrhization is dependent on external factors, such a categorization would be misguiding.

RESULTS

Among the 101 Ecuadorian fern samples, 85 species from 10 families were represented (Table 1). A total of 63 samples were infected by mycorrhizal fungi. AMF occurred in 19 species (22.35%) represented by 19 samples, and 36 species (42.35%) represented by 44 samples were infected by dark DSE (Table 2). Identified DSE always turned out to be ascomycetes that probably form a mycorrhizal association similar to the ericoid mycorrhiza (Schmid *et al.*, 1995; Kottke, 2002). Since it was not possible to process all specimens in question adequately, we retain the more general term DSE in the following

passages. The roots of 35 samples were free of evident fungal infection. Three specimens (*Arachniodes denticulata*, *Elaphoglossum lloense*, *Micropolypodium sp.*) had only a weak peripheral infection by DSE. They were regarded as dubious and are included in the non-mycorrhizal species (35.30% of the species). Mixed infections cannot be confidently reported.

AMF were found in 29.10% and 28.57% of the terrestrial and saxicolous species, respectively, but only in 3.45% of the epiphytes (Table 2). DSE showed a similar presence in terrestrial and saxicolous species (30.91% and 28.57%, respectively), but they dominated over AMF in the epiphytic species with 58.62%.

Hymenophyllaceae were represented with 18 species in our sample and showed a high presence of mycorrhization (78%). The mainly epiphytic species of *Hymenophyllum* were colonized by DSE (80%), whereas the predominantly terrestrial or saxicolous species of *Trichomanes* s.l. (*Trichomanes*, *Abrodyctium*) had more cases of AMF infection (50%). One unidentified *Trichomanes* grew epiphytically and had DSE like the epiphytic *Hymenophyllum* species. The only terrestrial *Trichomanes* s.l. with DSE was *Trichomanes dactylites* Sodiro.

Grammitid ferns (Polypodiaceae; Schneider *et al.*, 2004, Smith *et al.*, 2006), represented by 24 species, had an infection rate of 75%. Only ascomycetes (i.e., DSE) were found as fungal partner, even in terrestrial and saxicolous species (L. Pazmiño, unpubl. data). Non-grammitid Polypodiaceae were completely free of evident fungal infections.

Among the 23 species of *Elaphoglossum*, we found only 12 (52.20%) with fungal infection. DSE accounted for 75% of the infections.

The remainder of the investigated species showed mycorrhizal associations as was more or less expected from previous accounts. All three species of *Asplenium* (Aspleniaceae) were terrestrial and free of fungal infection. Of the two terrestrial species of *Blechnum* (Blechnaceae), only one had a low AMF infection. The investigated Pteridaceae showed a medium to strong infection by AMF (2 species, 100% infection).

Although they have been cited as examples for high infection rates (Boullard, 1958, 1979), only 50% of the species in the Cyatheaceae and 40% of the species in the Gleicheniaceae had mycorrhizal associations (Table 1). However, the exclusive colonization by AMF could be confirmed in both families.

Our sample size was not sufficient for a statistical analysis of changing mycorrhization along an elevational gradient. The localities of the samples are included in Table 1 for future studies focusing on this topic, which may want to include the data presented here.

Discussion

The overall infection by confirmed and putatively mycorrhizal fungi among our samples was 62.38% (64.70% at the species level). These percentages are lower than those reported for angiosperms or land plants in general. Trappe

TABLE 1. Investigated samples. Abbreviations: t = terrestrial, e = epiphyte, s = saxicolous; AMF = arbuscular mycorrhizal fungi; DSE = dark septate endophytes; ? = dubious record. Localities: A) Gualaceo-Limon road (3100–3300 m), B) mountain pass El Tiro (2600–2800 m), C) Cerro Toledo (2900–3100 m, Prov. Loja), D) Reserva Biológica San Francisco (1800–2600 m), E) Reserva Cajanuma (2750 m), F) Reserva Tapichalaca (2450–2650 m), G) the Campamento Indígena Shaimi (900–1200 m).

Species	Sub-strate	Fungal infection (%)	Type of infection	Collection	Loc.
Aspleniaceae					
Asplenium auritum Sw.	t	-	-	Lehnert M. 1473	F
Asplenium hallii Hook.	t	-	-	Lehnert M. 1395	B
Asplenium serra Langsd. & Fisch.	t	-	-	Lehnert M. 1394	B
Blechnaceae					
Blechnum schomburgkii (Klotzsch) C. Chr.	t	5–25	AMF	Lehnert M. 1484	D
Blechnum sp. 1	t	-	-	Lehnert M. 1440	D
Cyatheaceae					
Alsophila conantiana Lehnert	t	5–25	AMF	Lehnert M. 1414	B
Cyathea bipinnatifida (Baker) Domin	t	-	-	Lehnert M. 1438	D
Cyathea dudleyi R. M. Tryon	t	5–25	AMF	Lehnert M. 1550	D
Cyathea hybrid	t	-	-	Lehnert M. 1434	D
Cyathea obnoxia Lehnert	t	-	-	Lehnert M. 1470	F
Cyathea peladensis (Hieron.) Domin	t	5–25	AMF	Lehnert M. 855	D
Dryopteridaceae					
Arachniodes denticulata (Sw.) Ching	t	<5	DSE?	Lehnert M. 996	F
Elaphoglossum antisanae (Sodiro) H. Christ	e	-	-	Lehnert M. 1485	D
Elaphoglossum argyrophyllum (Sodiro) R. C. Moran, comb. ined.	e	-	-	Lehnert M. 1490	D
Elaphoglossum deltoideum (Sodiro) H. Christ	t	-	-	Lehnert M. 1460	C
Elaphoglossum deltoideum (Sodiro) H. Christ	t	-	-	Lehnert M. 1463	C
Elaphoglossum dendricola (Baker) H. Christ	t	-	-	Lehnert M. 1458	C
Elaphoglossum engelii (H. Karst.) H. Christ	t	25–75	DSE	Lehnert M. 1457	C
Elaphoglossum erinaceum (Fée) T. Moore	e	5–25	DSE	Lehnert M. 1387	B
Elaphoglossum glossophyllum Hieron.	e	-	-	Lehnert M. 1551	D
Elaphoglossum glossophyllum Hieron.	e	5–25	DSE	Lehnert M. 1552	D
Elaphoglossum guamanianum (Sodiro) C. Chr.	e	25–75	DSE	Lehnert M. 1493	D
Elaphoglossum heteromorphum (Klotzsch) T. Moore	t	5–25	AMF	Lehnert M. 1462	C

TABLE 1. Continued.

Species	Sub-strate	Fungal infection (%)	Type of infection	Collection	Loc.
Elaphoglossum latifolium (Sw.) J. Sm.	e	25–75	AMF	Lehnert M. 1492	D
Elaphoglossum lloense (Hook.) T. Moore	e	<5	DSE?	Lehnert M. 1491	D
Elaphoglossum papillosum (Baker) H. Christ	t	5–25	AMF	Lehnert M. 1472	F
Elaphoglossum petiolosum (Desv.) T. Moore	e	5–25	DSE	Lehnert M. & N. Mandl 1441	B
Elaphoglossum productum Rosenst.	e	-	-	Lehnert M. 1553	D
Elaphoglossum quitense (Baker) C. Chr.	t	25–75	DSE	Lehnert M. 1459	C
Elaphoglossum sp. 1	e	5–25	DSE	Lehnert M. 1487	D
Elaphoglossum sp. 2	e	-	-	Lehnert M. 1486	D
Elaphoglossum sp. 3	t	-	-	Lehnert M. 1502	D
Elaphoglossum squarrosum (Klotzsch) T. Moore	t	5–25	AMF	Lehnert M. 1488	D
Elaphoglossum vulcanicum H. Christ	e	-	-	Lehnert M. 1475	F
Elaphoglossum yatesii (Sodiro) H. Christ	t	25–75	DSE	Lehnert M. 1461	C
Lastreopsis kilippii (Maxon) Tindale	t	25–75	AMF	Lehnert M. 980	F
Polystichum platyphyllum (Willd.) C. Presl	t	25–75	AMF	Lehnert M. 1412	B
Gleicheniaceae					
Sticherus brevitomentosus B. Øllg. & Østergaard	t	-	-	Lehnert M. 1480	F
Sticherus melanoblastus Østergaard & B. Øllg.	t	-	-	Lehnert M. 1549	D
Sticherus melanoblastus Østergaard & B. Øllg.	t	-	-	Lehnert M. 1476	F
Sticherus rubignosus (Mett.) Nakai	t	-	-	Lehnert M. 1268	F
Sticherus rubignosus (Mett.) Nakai	t	5–25	AMF	Lehnert M. 1478	F
Sticherus sp. 1	t	5–25	AMF	Lehnert M. 1479	F
Sticherus tomentosus (Cav. ex Sw.) A. R. Sm.	t	-	-	Lehnert M. 1477	F
Hymenophyllaceae					
Abrodictyum rigidum (Sw.) Ebihara & Dubuisson	t	25–75	AMF	Lehnert M. 1515	G
Hymenophyllum calodictyon Bosch	e	5–25	DSE	Lehnert M. 1443	B
Hymenophyllum cristatum Hook. & Grev.	e	5–25	DSE	Lehnert M. 1547	D
Hymenophyllum fucoides (Sw.) Sw.	e	5–25	DSE	Lehnert M. 1444	B
Hymenophyllum microcarpum Desv.	t	5–25	DSE	Lehnert M. 1494	D
Hymenophyllum multialatum C. V. Morton	e	25–75	DSE	Lehnert M. 1447	B
Hymenophyllum plumierii Hook. & Grev.	e	25–75	DSE	Lehnert M. 1362	B

TABLE 1. Continued.

Species	Sub-strate	Fungal infection (%)	Type of infection	Collection	Loc.
Hymenophyllum polyanthos (Sw.) Sw.	e	5–25	DSE	Lehnert M. 1445	B
Hymenophyllum sp. 1	e	-	-	Lehnert M. 1455	C
Hymenophyllum sp. 2	s	-	-	Lehnert M. 1566	A
Hymenophyllum trichomanoides Bosch	e	5–25	DSE	Lehnert M. 1446	B
Trichomanes cellulosum Klotzsch	t	5–25	AMF	Lehnert M. 1481	D
Trichomanes dactylites Sodiro	t	5–25	DSE	Lehnert M. 1501	D
Trichomanes elegans Rich.	s	5–25	AMF	Lehnert M. 1516	G
Trichomanes pellucens Kunze	t	5–25	AMF	Lehnert M. 1514	G
Trichomanes sp. 1	e	5–25	DSE	Lehnert M. 1483	D
Trichomanes sp. 2	s	-	-	Lehnert M. 1546a	G
Trichomanes sp. 3	t	-	-	Lehnert M. 1482	D
Loxomataceae					
Loxsomopsis pearcei (Maxon) Baker	t	>75	AMF	Lehnert M. 1056	F
Polypodiaceae [non-grammitids]					
Campyloneurum amphostenon Fée	t	-	-	Pazmiño L. s.n.	D
Niphidium albopunctatissimum Lellinger	t	-	-	Pazmiño L. s.n.	D
Pleopeltis percussa Hook. & Grev.	t	-	-	Pazmiño L. s.n.	D
Polypodiaceae [grammitids]					
Ceradenia farinosa (Forssk.) Kaulf.	e	25–75	DSE	Pazmiño L. s.n.	D
Ceradenia farinosa (Forssk.) Kaulf.	t	5–25	DSE	Pazmiño L. s.n.	D
Ceradenia glabra A. R. Smith & M. Kessler	e	5–25	DSE	Lehnert M. 1495	D
Cochlidium serrulatum (Sw.) L. E. Bishop	t	5–25	DSE	Lehnert M. 1467	C
Cochlidium serrulatum (Sw.) L. E. Bishop	e	5–25	DSE	Pazmiño L. s.n.	D
Enterosora parietina (Klotzsch) L.E. Bishop	e	-	-	Lehnert M. 1497	D
Grammitis paramicola L. E. Bishop	e	25–75	DSE	Pazmiño L. s.n.	D
Grammitis paramicola L. E. Bishop	t	25–75	DSE	Pazmiño L. s.n.	D
Lellingeria major (Copel.) A. R. Sm. & R.C. Moran	t	25–75	DSE	Lehnert M. 1466	C
Lellingeria major (Copel.) A. R. Sm. & R.C. Moran	e	25–75	DSE	Lehnert M. 1498	D
Lellingeria subsessilis (Baker) A. R. Sm. & R. C. Moran	e	-	-	Lehnert M. 1499	D
Lellingeria subsessilis (Baker) A. R. Sm. & R. C. Moran	e	25–75	DSE	Pazmiño L. s.n.	D
Lellingeria subsessilis (Baker) A. R. Sm. & R. C. Moran	t	5–25	DSE	Pazmiño L. s.n.	D

TABLE 1. Continued.

Species	Sub-strate	Fungal infection (%)	Type of infection	Collection	Loc.
Melpomene assurgens (Maxon) A. R. Sm. & R. C. Moran	e	25–75	DSE	Lehnert M. 1427	B
Melpomene erecta (C. V. Morton) A. R. Sm. & R. C. Moran	t	5–25	DSE	Lehnert M. 1570	A
Melpomene firma (J. Sm.) A. R. Sm. & R. C. Moran	e	25–75	DSE	Lehnert M. 1328	B
Melpomene gracilis (Hook.) A. R. Sm. & R. C. Moran	s	5–25	DSE	Lehnert M. 1569	A
Melpomene moniliformis (Lagasca ex Sw.) A.R. Sm. & R.C. Moran	e	5–25	DSE	Lehnert M. 1559	A
Melpomene moniliformis (Lagasca ex Sw.) A.R. Sm. & R.C. Moran	t	5–25	DSE	Lehnert M. 1510	E
Melpomene occidentalis Lehnert	t	-	-	Lehnert M. 1507	E
Melpomene occidentalis Lehnert	t	-	-	Lehnert M. 1508	E
Melpomene pseudonutans (Christ & Rosenst.) A.R. Sm. & R.C. Moran	s	5–25	DSE	Lehnert M. 1558	A
Melpomene pseudonutans (Christ & Rosenst.) A.R. Sm. & R.C. Moran	t	5–25	DSE	Lehnert M. 1464	C
Melpomene pseudonutans (Christ & Rosenst.) A.R. Sm. & R.C. Moran	e	25–75	DSE	Pazmiño L. s.n.	D
Melpomene sklenarii Lehnert	t	5–25	DSE	Lehnert M. 1465	C
Melpomene wolfii (Hieron.) A. R. Sm. & R. C. Moran	e	25–75	DSE	Pazmiño L. s.n.	D
Melpomene wolfii (Hieron.) A. R. Sm. & R. C. Moran	t	5–25	DSE	Pazmiño L. s.n.	D
Micropolypodium sp. 1	t	<5	DSE?	Pazmiño L. s.n.	D
Micropolypodium sp. 1	e	5–25	DSE	Pazmiño L. s.n.	D
Terpsichore lanigera (Desv.) A. R. Sm.	e	-	-	Lehnert M. 1496	D
Terpsichore leucosticta (J. Sm.) A. R. Sm.	t	5–25	DSE	Lehnert M. 1509	E
Terpsichore semihirsuta (Klotzsch) A. R. Sm.	t	5–25	DSE	Lehnert M. 1511	E
Pteridaceae					
Pteris muricata Hook.	t	5–25	AMF	Lehnert M. 1571	B
Pterozonium brevifrons (A. C. Sm.) Lellinger	s	25–75	AMF	Lehnert M. 1435	D
Thelypteridaceae					
Thelypteris minutula C. V. Morton	s	-	-	Lehnert M. 1337	B

TABLE 2. Distribution of the 85 investigated species onto the registered categories: total numbers are followed by percentages per life forms (columns) and infection types (rows) in brackets. Abbreviations are the same as in Table 1; NM = non-mycorrhizal; ** six species occurred on more than one substrate, adding 7% to the total count and 20% to NM.

Species	All	t	e	s
total	85 (100/100**)	55 (100/64.71**)	29 (100/34.12**)	7 (100/8.24**)
AMF	19 (22.35/100)	16 (29.10/84.21)	1 (3.45/5.26)	2 (28.57/10.53)
DSE	36 (42.35/100)	17 (30.91/47.22)	17 (58.62/47.22)	2 (28.57/5.55)
NM	30 (35.30/100**)	22 (35.29/73.33**)	11 (37.93/36.67**)	3 (42.86/10.00**)

(1987) estimated that 82% of angiosperms host mycorrhizae; Wang and Qiu (2006) concluded that 80% of all land plants are mycorrhizal.

Studies focusing on ferns and lycophytes found similar results to ours. Values gathered from literature (Boullard, 1958; Cooper, 1976; Berch and Kendrick, 1982, Iqbal *et al.*, 1981, Gemma *et al.*, 1992, Lesica and Antibus, 1990, Moteetee *et al.*, 1996; Ragupathy and Mahadevan, 1993, Schmid *et al.*, 1995, Muthukumar and Udaiyan, 2000; Zhao, 2000; Zhang *et al.*, 2003) sum up to 68% of general fungal colonization and to 53% of AMF in ferns and lycophytes (M. Lehnert, unpubl. data). Wang and Qiu (2006), considering only AMF, found a comparable 52% of the species of ferns and lycophytes to be mycorrhizal.

Despite the congruence in general mycorrhizal infection, our survey found AMF in only 22.35% of the species, including 29.10% of the terrestrial, 28.57% of the saxicolous species, and only 3.45% of the epiphytes. In contrast, DSE showed a similar presence in terrestrial and saxicolous species (30.91% and 28.57%), but they dominated over AMF in the epiphytic species with 58.62%.

The discrepancy in AMF percentages between our study and the average is likely due to our selective sampling. We laid the focus on predominantly epiphytic taxa, and although we still examined more terrestrial than epiphytic samples, we evidently included a higher percentage than previous studies. The epiphytic habitat is rarely colonized by AMF because their spores are not easily dispersed from the soil. Furthermore, most AMF are dependent on their host, requiring the presence of a facultatively mycorrhizal plant for successfully establishing the symbiosis on a chorophyte (Janos, 1993). Thus the low presence of AMF in epiphytes (3.45%) is not surprising. DSE, however, have spores that get airborne and are thus more likely to contact the roots of epiphytic plants. Epiphytic plant species are well known to suffer from nutrient shortages and should greatly benefit from a fungal symbiont (Lesica and Antibus, 1990). If DSE are excluded in surveys as potential mycorrhizal partners in ferns and lycophytes (Lesica and Antibus, 1990; Michelsen, 1993), the recorded mycorrhization is low or absent, in our case only 22.4%. If they are regarded as mycorrhizae, the mycorrhization level will increase (Schmidt *et al.*, 1995; Kottke, 2002), in our case to 42.35%. Overall, the degree of

infection by DSE was higher in our study than in any other previous study on ferns and lycophytes.

Beyond these general patterns, it is worthwhile to focus on individual study groups. The Hymenophyllaceae nicely mirror the general distribution pattern of the fungal infections. Terrestrial and saxicolous species have predominantly AMF, whereas DSE prevail in epiphytes. Gammitid ferns (Polypodiaceae), however, have almost exclusively DSE, no matter if they grew as epiphytes or as terrestrials. This apparent conflict with the general trend is due to the microhabitats inhabited by the species. The investigated terrestrial grammitid ferns usually grew in thick moss cushions like their epiphytic kin and by this means under very similar ecological conditions, which may lead to maintaining the type of mycorrhiza. Furthermore, most of the species sampled as terrestrials are either potentially epiphytic or closely related to epiphytic species. Only the samples of *Melpomene occidentalis* Lehnert rooted directly in mineral soil and showed no fungal infection. Opposed to this, the samples of eleven terrestrial and epiphytic species of non-grammitid Polypodiaceae from the investigated area are free of fungal infections, which is congruent with previous reports (Lesica and Antibus, 1990; Schmid *et al.*, 1995). Since grammitid ferns represent a clade nested deeply within the Polypodiaceae, it is likely that the original condition in the family is a lack of mycorrhization and that mycorrhization has been secondarily regained in grammitid ferns. Apparently, this symbiosis was developed with DSE rather than with glomeromycetes. A similar situation of loss of AMF mycorrhization and secondary gain of DSE mycorrhization, also related with shifts between the terrestrial and epiphytic habitat, has been reported in liverworts (Kottke and Nebel, 2005).

The genus *Elaphoglossum* showed no clear correlation between the types of substrate and fungal infections. The genus *Asplenium* is not very diverse or abundant in the study sites and occurred only on the lower slopes where nutrients are accumulated (Gradstein *et al.*, 2008). The absence of mycorrhizae in our samples may be related to the improved availability of nutrients at their microhabitats. Previous studies found generally low infection rates in the Aspleniaceae (e.g., Boullard, 1958) and often varying results within a species, indicating that most species may be only facultatively mycorrhizal.

Gleicheniaceae are usually axiomatic for strong presence of mycorrhizae (100%; Boullard, 1958, 1979). It is assumed that this affects both their ability to grow on nutrient deficient soils and their inability to be transplanted and cultivated. Surprisingly, we found only 40% of our samples infected by AMF. Their root samples, however, were difficult to prepare because of a tough texture and dark, persistent cortical colorants. The necessary clearing with hydrogen peroxide may have affected the colourability of fungal hyphae with dye. Possibly a higher percentage of fungal infections was present but not detectable in our samples of Gleicheniaceae.

Our results for the Cyatheaceae are much lower (50% of specimens infected) than the results of previous surveys (100% of specimens infected; Boullard, 1958; Hepden, 1960). The tree ferns (families Cyatheaceae and Dicksoniaceae)

bear the difficulty of acquiring fine roots from the compact subterranean root system that many species develop. Aerial roots from the trunks are easier to harvest but are expected to lack mycorrhizae because they are less likely to get in contact with inoculum of soil fungi. In order to bypass this sampling artefact, the plants included in this study were either small species or young plants of easily assignable larger species, which can be uprooted with most of their roots. One explanation for the low infection rate could be that these juvenile plants of *Cyathea* are less dependent on mycorrhizae than mature plants. The trunk-less tree ferns dwell in the shade where these often sun-loving species are under lesser drought stress but presumably achieve only a part of their potential photosynthetic rate. The profits of better supply with water and micronutrients may not compensate the cost of sharing assimilates with symbiotic fungi.

We are aware that negative results in any species here included do not exclude the potential occurrence of mycorrhiza in other individuals of the same species. We aim to widen our sample size and want to include conspecific samples from sites with different substrate chemistry. This should allow us not only to distinguish between facultative and obligatory mycorrhizae but also about the conditioning factors.

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Differences In Post-Emergence Growth Of Three Fern Species Could Help Explain Their Varying Local Abundance

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ABSTRACT.—Despite the large number of comparative studies on species with different distribution and abundance, no clear general pattern of attributes explaining species' rarity has yet been found. The relationship between different life-history traits of a species and abundance tend to be conditional and context dependent. We were interested in whether the local relative population density of three fern species in Estonia is related to post-emergence growth of their young sporophytes, i.e., that the locally abundant species, *D. carthusiana*, has the highest vegetative growth in its first growth periods and the two less abundant species, *D. dilatata* and *D. expansa*, have lower. We were also interested in differences between generative traits of young sporophytes of three species, specifically in the number of spores. We grew the species in a garden experiment for two vegetation periods, 2004–2005, until the first sporulation. The relative population density of the three *Dryopteris* species was related to the relative post-emergence growth of the species. The most abundant species *D. carthusiana*, exhibited the highest values of vegetative growth parameters in the first growth period. The less abundant *D. dilatata* and *D. expansa* both had shorter fronds, shorter intensive growth periods and lower leaf elongation rates. *Dryopteris dilatata* had a different vegetative growth strategy compared to the other two species; it differed in timing of intensive growth of frond length and increase of frond number and had the lowest values of generative parameters among the three species.

KEY WORDS.—*Dryopteris*, Post-emergence growth, Rarity

Ecology is aimed at detecting factors and processes that control the relative abundance and distribution of species (Kunin and Gaston, 1997; Crawley, 1997). Understanding why some species are more common than others provides us with basic information about the distribution and regional dynamics of different species. Such understanding is essential for the practical conservation and management of rare species, i.e., species with a low relative abundance/distribution at continental, and particularly at regional and local levels.

One possible approach for investigating the mechanisms behind rarity is through the comparison of taxa with contrastingly different distribution and abundance patterns (e.g., Baskauf and Eicmeier, 1994; Sultan, 2001; Simon and Hay, 2003; Pohlman *et al.*, 2005). The study of pairs or even larger numbers of closely related taxa with common genetic heritage may more easily reveal factors limiting rare species (Baskin and Baskin, 1986; Silvertown and Dodd,

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1996; Gitzendanner and Soltis, 2000). Despite a large number of comparative studies on the subject (e.g., reviewed in Bevill and Louda, 1999; Binney and Bradfield, 2000; Brown *et al.*, 2003; Rymer *et al.*, 2005), no clear pattern of general attributes or one specific feature explaining species' rarity has yet been found. Relationships between different life-history traits of a species and abundance tend to be conditional and context dependent (Murray *et al.*, 2002).

Several recent studies have focused on the relative importance of dispersal and environmental determinants of fern distribution. Evidence has been found that habitat availability, at a local scale (Richard *et al.*, 2000; Wild and Gagnon, 2005) and a regional scale (Guo *et al.*, 2003), and not dispersal capability is responsible for fern distribution. Karst *et al.*'s study (2005) at two contrasting local spatial scales (local mesoscale and local fine) showed that fern distribution at the local mesoscale (135–3515 m) was linked to environmental factors, but at the local fine scale (4–134 m); both dispersal and abiotic environment were jointly responsible for fern distribution.

Comparative studies of the different life phases of fern ecology (spores, gametophytes and sporophytes) have shown the different amplitude of the abiotic factors under study. Although this amplitude is usually broader in the case of spores (compared with gametophytes; Hill, 1971; Prada *et al.*, 1995) and gametophytes (compared with sporophytes; Sato and Sakai, 1981), the persistence of fern species in a habitat is possible only if the realized niches of spores, gametophytes and sporophytes match. A habitat that meets requirements of gametophytes or young sporophytes may be less or not at all suitable for mature sporophytes. Consequently the early period of sporophyte generation, then vascular sporophyte emergences from a small non-vascular gametophyte (Page, 2002) is extremely important in the life of a fern species. For this reason traits of post-emergence growth (Leishman, 1999) of young sporophytes could be particularly important for the performance of a species and determine distribution. The degree of influence of resource availability in the pertinent location on the success of early growth of young sporophytes' is known to be high (Grime, 1985) and may to a great extent depend on competitive pressure of surrounding neighbors (Cousens, 1981; Grime *et al.*, 1988; Rünk *et al.*, 2006).

The current study is a part of a larger project investigating the possible reasons of different regional frequency and local abundance of three closely related co-occurring fern species: *Dryopteris carthusiana*, *Dryopteris expansa* and *Dryopteris dilatata*. *Dryopteris carthusiana* is common in Estonia; *D. expansa* is distributed in scattered localities throughout Estonia, while *D. dilatata* is rare, being close to its north-eastern distribution limit. According to our earlier study (Rünk *et al.*, 2004); the different competitive abilities of *D. carthusiana* and *D. expansa* might help explain their different relative regional frequency, but not in the case of *D. dilatata*, which is near its distribution border as tolerant to competition as the most frequent *D. carthusiana*. Climatic factors are a likely limitation to distribution of *D. dilatata* in Estonia. The northern distribution limit of this species is approximately 300 km from Estonia, in southern Finland (Hultén and Fries, 1986), and shadows the

isothermal line along which the coldest month is between 5 and 8°C (Boucher, 1987). Still, the particular mechanism behind climatic restrictions remains open to debate.

The results of field survey of the three species on permanent plots showed the higher local relative population density of *D. carthusiana* compared to *D. dilatata* and *D. expansa* (Rünk *et al.*, 2006). The order of the species' rankings could be explained by the competitive ability of the three fern species.

Therefore we hypothesized that the local relative population density of the three fern species is related to the success of post-emergence growth of their young sporophytes, i.e., that comparatively more abundant species have the highest vegetative growth in their first growth periods. We were also interested in whether there were differences in the generative traits of the three species' young sporophytes, and erected the hypothesis that *D. dilatata* had the lowest number of spores than *D. carthusiana* and *D. expansa*.

Material and Methods

Study species.—The three species studied are closely related from an evolutionary point of view (Gibby and Walker, 1977) and are morphologically similar (Fraser-Jenkins and Reichstein, 1984; Page, 1997). All three species are medium-sized, rhizomatous, herbaceous plants with 3-pinnate fronds and orbicular sori covered with reniform indusia (Fraser-Jenkins, 1993). Tetraploid (2n = 164) *Dryopteris carthusiana* (Vill.) H.P. Fuchs is the most common of the three species, and can be found throughout Europe, North America, West and Southeast Asia (Hultén and Fries, 1986; Fraser-Jenkins, 1993). *Dryopteris expansa* (C. Presl) Fraser-Jenkins and Jermy can also be found in North America and Asia. Tetraploid (2n = 164) *Dryopteris dilatata* (Hoffm.) A. Gray is distributed mostly in Western and Central Europe (Hultén and Fries, 1986; Fraser-Jenkins, 1993). Diploid (2n = 82) *D. expansa* is mainly restricted to mountainous regions of Europe, and has a more northerly and easterly distribution than *D. dilatata* (Fraser-Jenkins and Reichstein, 1984; Hultén and Fries, 1986). Piękoś-Mirkova (1991) found *D. expansa* at 2098 meters above sea level, above the timberline in the Poland's Tatra Mountains. In Scandinavia, the distribution limit of *D. expansa* is the northernmost of the three species (Jonsell, 2000). In Western and Central Europe, *D. dilatata* is a more common species than *D. expansa* (Fraser-Jenkins and Reichstein, 1984; Page, 1997). In Estonia the opposite is true; *D. expansa* is distributed in scattered localities throughout Estonia (Kukk and Kull, 2005), while *D. dilatata*, close to its north-eastern distribution limit (Page, 1997; Jonsell, 2000), is rare. *Dryopteris carthusiana* possesses the highest regional frequency of the three species, and is evenly distributed across the country. Similarly, the local abundance (population density) of *D. carthusiana* is the highest among the three species (Rünk *et al.*, 2006). According to the Atlas of the Estonian Flora (Kukk and Kull, 2005), in which Estonia is divided into a grid of 513 (6 × 10 minute squares), D. carthusiana was recorded in 441, *D. expansa* in 145 and D. dilatata in 20 of the squares. While *D. expansa*, like *D. carthusiana*, is distributed

evenly, most of *D. dilatata* populations are situated in the northern and western part of the country. In Estonia all the species can be found growing in mesic woodlands (Rünk, 2002), mostly in mixed populations.

All three fern species (*D. carthusiana*, *D. dilatata* and *D. expansa*) are sexually reproducing species (Manton, 1950) with sporangia that contain 64 spores (Widén *et al.*, 1967; Schneller, 1975; Fraser-Jenkins and Reichstein, 1984) with a similar size per sporangium (Piękoś-Mirkova, 1979; Seifert, 1992). Species nomenclature follows Fraser-Jenkins (1993).

Experimental design.—Vegetative growth, reproduction, morphology and biomass were assessed in a common garden experiment conducted in 2004 and 2005. Spores of all fern species were collected in the wild in July 2003 and stored in a refrigerator (at 2 ± 1°C) until the beginning of the experiment. The substrate used for spore germination was sterilized and consisted of 3 parts horticultural peat and 1 part fine-grade sand. Spores were sown on October 20, 2003 and sporophytes emerged in March 2004. Young sporophytes were planted, nine evenly spaced per plastic box (12 × 8 × 8 cm deep), on May 16, 2004. The specimens were replanted individually in plastic pots (10 cm diameter, 8 cm deep) on August 2. Initially all three species were represented by 60 individuals, but for the final harvest and analysis, 15 individuals per species were randomly selected.

The soil mixture used for receiving sporophyte plants consisted of 4 parts horticultural peat and 1 part fine-grade sand. The boxes were placed in a greenhouse at 22 ± 2°C with a photoperiod of 12:12 h (fluorescent light: daylight tubes, photon flux density 40 μmol $s^{-1}m^{-2}$) and watered as needed to keep the soil moist. On August 10 the pots were relocated to the experimental garden and grown in shaded light for another 14 months. In order to minimize possible differences in illumination, the positions of all pots were changed weekly. To imitate the species' natural Estonian environment a screen with a shade value of 65% was used, as all three species can be found growing mainly in mesic woodlands. Shade treatment was provided using a screen made of aluminum-coated shade cloth (spectrum neutral; Ludvig Svensson, Kinna, Sweden). During the winter of 2004/2005, plants were covered with horticultural peat imitating fallen leaves and their decayed remnants.

The experimental garden was located in Tartu (58°21′25″N, 26°42′5″E, 68 meters a.s.l.), in south-eastern Estonia, where the average annual temperature is 5.0°C and the average amount of annual precipitation is 550 mm (Jaagus, 1999).

Data collection.—During the two growing seasons, a total of nine measurements were conducted every 28–34 days. Five measurements were made in 2004 (on June 9, July 9, August 9, September 10, October 8) and four in 2005 (on June 22, July 27, August 25, September 30), the first measurement of each year occurring when the fronds had rolled out and the last just before the first autumn frost. For each individual, the number of fronds was counted and the length of the longest frond was measured. In generative individuals, the number of fertile (spore-bearing) fronds was also counted. In the case of the length of the longest frond the frond was measured to the nearest millimeter on

each individual fern between the base of the stipe (stalk of the frond) and the tip. Those measurements allowed us to calculate leaf elongation rate (LER) and frond number increase rate (NIR).

Leaf elongation rate (LER, mm/day) were calculated using the following basic equation:

$$\mathrm{LER} = \frac{(M_{n+1} - M_n)}{D} \quad (1)$$

where M_{n+1} is the current measurement in millimeters; M_n is the previous measurement in millimeters and D is the number of days between measurements.

Frond number increase rate (NIR, number of fronds/day) was calculated using the following basic equation:

$$\mathrm{NIR} = \frac{(F_{n+1} - F_n)}{D} \quad (2)$$

where F_{n+1} is current measurement (number of fronds); F_n is the previous measurement (number of fronds); D is the number of days between measurements.

LER and NIR were calculated for all seven time intervals between the measurements; four in 2004 (June, July, August and September) and three in 2005 (July, August and September). In generative individuals, the number of fertile (spore-bearing) fronds was also counted.

After the final harvest in October 2005, fronds, rhizomes and roots were separated and dried at 75°C for 48 hours. Biomass fractions were determined by weighing the parts separately. The length of all fronds and frond laminae (the leafy part of the frond) were measured to the nearest millimeter before the final harvest. The length of the stipe was obtained by subtracting lamina length from frond length. Lamina area and lamina area (pinnae) covered with sori were measured using a scanner (ScanJet5p), DeskScan II 2.9, and Pindala 1.0 software (designed by I. Kalamees, Eesti Loodusfoto, Tartu, Estonia). Specific leaf area (SLA) was calculated as lamina area (cm^2) per unit of lamina dry mass (g).

Statistical analysis.—Differences in and the timing of vegetative growth (length of the longest frond and the number of fronds) during the both growth periods were tested separately for each year with repeated measures of ANOVA (using the Statistica software version 6.0; StatSoft Inc., 1998) with the species (three levels) as fixed factors and measurement time (five levels in 2004 and four levels in 2005) as a repeated factor.

Differences in vegetative growth rate, LER and NIR, between *D. carthusiana*, *D. dilatata* and *D. expansa* during the growth periods in the years 2004 and 2005 were tested separately for each year with repeated measures of ANOVA with the species (three levels) as fixed factors and period of time between measurements (four levels in 2004 and three levels in 2005) as a repeated measurement factor.

TABLE 1. Results of repeated measures ANOVA: effects of species, measurement time and their interaction on the length of the longest frond and on the number of fronds of *Dryopteris carthusiana, D. expansa* and *D. dilatata* in 2004 and in 2005.

Source of variation	Species			Time			Species*time		
	Df	F	P	Df	F-ratio	P	Df	F	P
Length of the longest frond in 2004	2	5.315	0.009	4	414.98	<0.000	8	12.63	<0.000
Length of the longest frond in 2005	2	9.448	<0.000	3	532.35	<0.000	6	5.937	<0.000
Number of fronds in 2004	2	21.88	<0.000	4	418.52	<0.000	8	4.865	<0.000
Number of fronds in 2005	2	11.88	<0.000	3	251.58	<0.000	6	1.508	0.181

Differences in the length of the longest frond and the number of fronds at the end of both growth periods and other morphological, biomass and reproductive parameters between *D. carthusiana*, *D. dilatata* and *D. expansa* at the end of the experiment were tested by one-way ANOVA with the species (three levels) as fixed factors. In the case of LER and NIR the equation $X' = \sqrt{X} + \sqrt{X} + 1$ was used for transformation (Zar, 1999). All other variables were log transformed, except in the case of relative biomass allocation, for which the data (as proportions) was arcsine square root transformed.

Differences between mean number and length of fertile and sterile fronds among species were tested by Students' t-test. The significance of the differences among all other parameters means was estimated with a Tukey HSD multiple-comparison test with a 0.05 significance level (Sokal and Rohlf, 1995).

RESULTS

Vegetative Growth Traits

Vegetative growth and timing of the vegetative growth.—During both growth periods in 2004 and 2005, there were differences in length of the longest frond and in number of fronds between the three species (Table 1). *Dryopteris carthusiana* and *D. dilatata* were characterized by longer fronds and by a higher number of fronds than *D. expansa*; all differences were significant except in the case of the length of fronds between *D. dilatata* and *D. expansa* in 2004. There were also differences in the timing of vegetative growth between the species in 2004 and 2005 (Table 1), except in the case of the number of fronds in 2005. In 2004, *D. carthusiana* had the longest period of intensive growth when the increase in number of fronds and length of the longest frond between measurements were significant. The production of new fronds and the growth of the longest frond continued until September. *Dryopteris expansa* had the shortest period of intensive growth of the three species; the number of leaves increased until August and the length of the longest frond increased only until July. *Dryopteris dilatata* produced new fronds even in September, however the growth period of the longest frond matched that of *D. expansa*; it took place only in June.

TABLE 2. Results of repeated measures ANOVA: effects of species, period of time between measurements and their interaction on the LER and NIR of *Dryopteris carthusiana*, *D. expansa* and *D. dilatata* in 2004 and 2005.

Source of variation	Species			Time period			Species* time period		
	Df	F	P	Df	F	P	Df	F	P
LER 2004	2	22.66	<0.000	3	42.73	<0.000	6	4.704	<0.000
LER 2005	2	8.181	0.001	2	70.03	<0.000	4	0.848	0.499
NIR 2004	2	7.000	0.003	3	10.15	<0.000	6	1.638	0.144
NIR 2005	2	11.81	<0.000	2	17.53	<0.000	4	1.264	0.291

LER (leaf elongation rate) and NIR (fronds number increase rate).—Differences in LER (Table 2, Fig. 1) were more distinct than in growth of the longest frond or number of fronds; *D. carthusiana* had significantly the highest LER in 2004 and *D. dilatata* in 2005; the differences between the other two species were non-significant in both years. The timing of LER was different only in 2004; *D. carthusiana* had significantly higher LER in August 2004, compared to the two other species, and in July 2004, compared to *D. dilatata*. There were also differences in LER between 2004 and 2005. At the beginning of the experiment in 2004, LER of *D. expansa* and *D. dilatata* dropped during July, while in the case of *D. carthusiana*, high LER continued up to September.

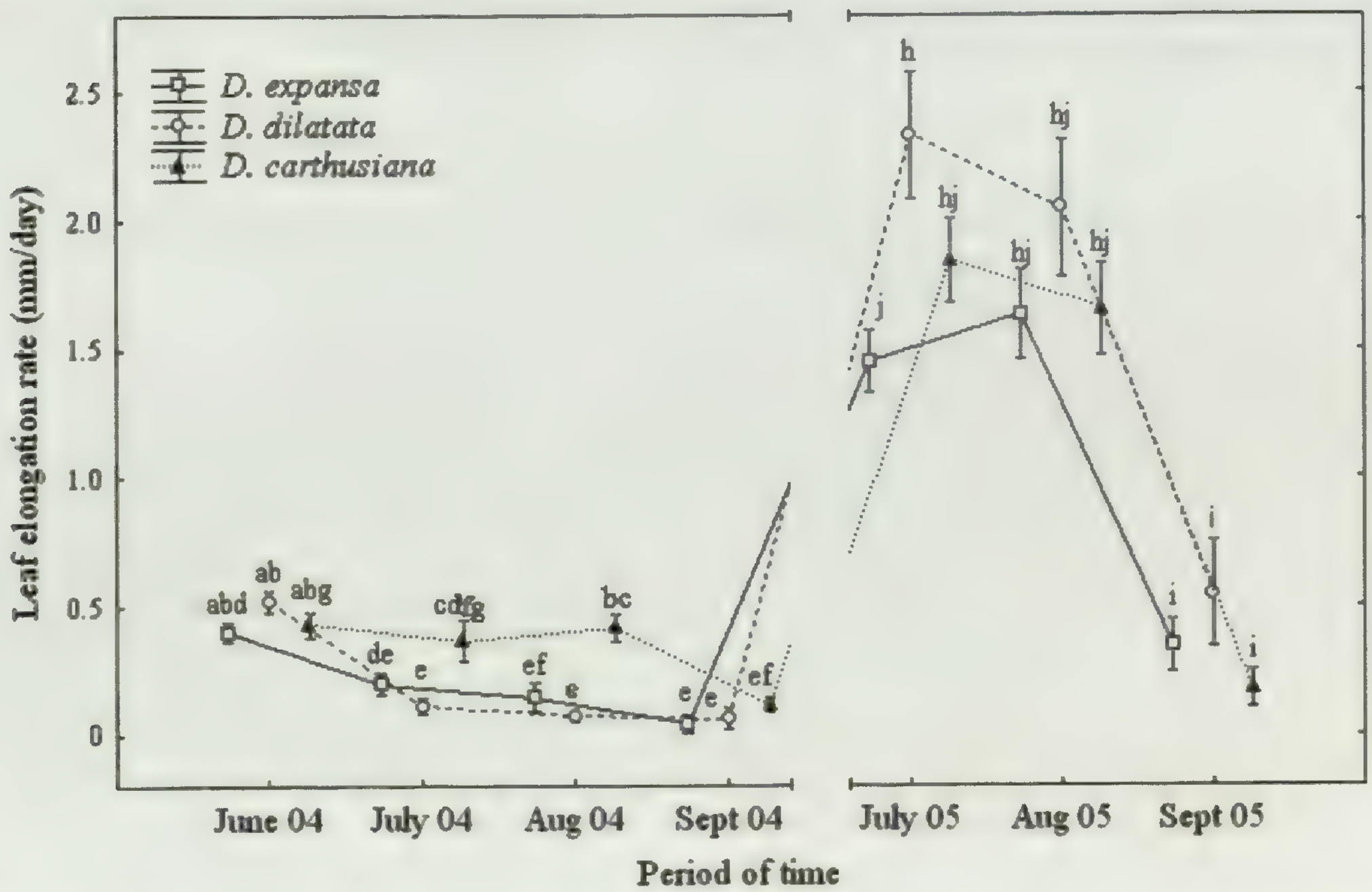

FIG. 1. Mean ± SE of the LER (mm/day) of the longest frond of *Dryopteris expansa*, *D. dilatata* and *D. carthusiana* in June (09/06–09/07), July (09/07–09/08), August (09/08–10/09), September (10/09–08/10) 2004 and in July (22/06–27/07), August (27/07–25/08), September (25/08–30/09) 2005. Whiskers with the same letter are not significantly different ($P < 0.05$, Tukey test; separately for 2004 and 2005). X-axis breaks between the results of different analysis.

TABLE 3. Results of one-way ANOVA: effects of species on the morphological, biomass and reproductive traits of *Dryopteris carthusiana*, *D. expansa* and *D. dilatata* at the end of the first growth period (October 2004) and at the final harvest (September 2005).

	Species (Df = 2)	
Source of variation	F	P
October 2004		
Length of the longest frond	13.60	<0.000
No of fronds	13.52	<0.000
September 2005		
Length of the longest frond	11.72	<0.000
Mean frond length	6.653	0.003
Mean lamina length	4.508	0.017
Mean stipe length	23.93	<0.000
No of fronds	24.54	<0.000
No of fertile fronds	3.754	0.033
No of sterile fronds	12.79	<0.000
Total mass	13.85	<0.000
Rhizome mass	5.968	0.005
Root mass	13.35	<0.000
Frond mass	17.67	<0.000
Relative biomass allocation to lamina	14.20	<0.000
Relative biomass allocation to rhizome	13.97	<0.000
Lamina area	22.91	<0.000
SLA	3.413	0.042
Pinnae area covered with sori	5.472	0.009

In 2005, LER of all three species was the highest at the beginning of the vegetation period and fell significantly in September, at the end of the growth period.

The differences in NIR (Table 2) were similar in both vegetation periods. The increase in number of fronds of *D. carthusiana* and *D. dilatata* was higher than that of *D. expansa*.

Morphological parameters and biomass allocation at the end of growth periods.—The effects of species on the morphological traits and biomass allocation of *D. carthusiana*, *D. expansa* and *D. dilatata* are summarized in Table 3. In October 2004, by the end of the first growth period *D. carthusiana* had the tallest plants (the longest fronds); while two other species were shorter (Fig. 2). By the end of the second growth period (September 2005) *D. dilatata* and *D. carthusiana* both had the longest fronds (Fig. 2). *Dryopteris carthusiana* also had longer fronds (Fig. 2) and stipes than the other two species, and had longer laminae per individual at the end of the experiment (at the final harvest) than *D. expansa*. *Dryopteris expansa* had fewer fronds than other two species in both growth periods (Fig. 3). Fertile fronds of all three species were significantly longer than their sterile fronds (t-test for *D. carthusiana*: $t = -15.42$, $Df = 12$, $P = <0.000$; *D. dilatata*: $t = -7.010$, $Df = 11$, $P = <0.000$ and *D. expansa*: $t = -16.82$, $Df = 13$, $P = <0.000$). *Dryopteris carthusiana* and *D. dilatata* both had significantly higher biomass in regard to all fractions studied

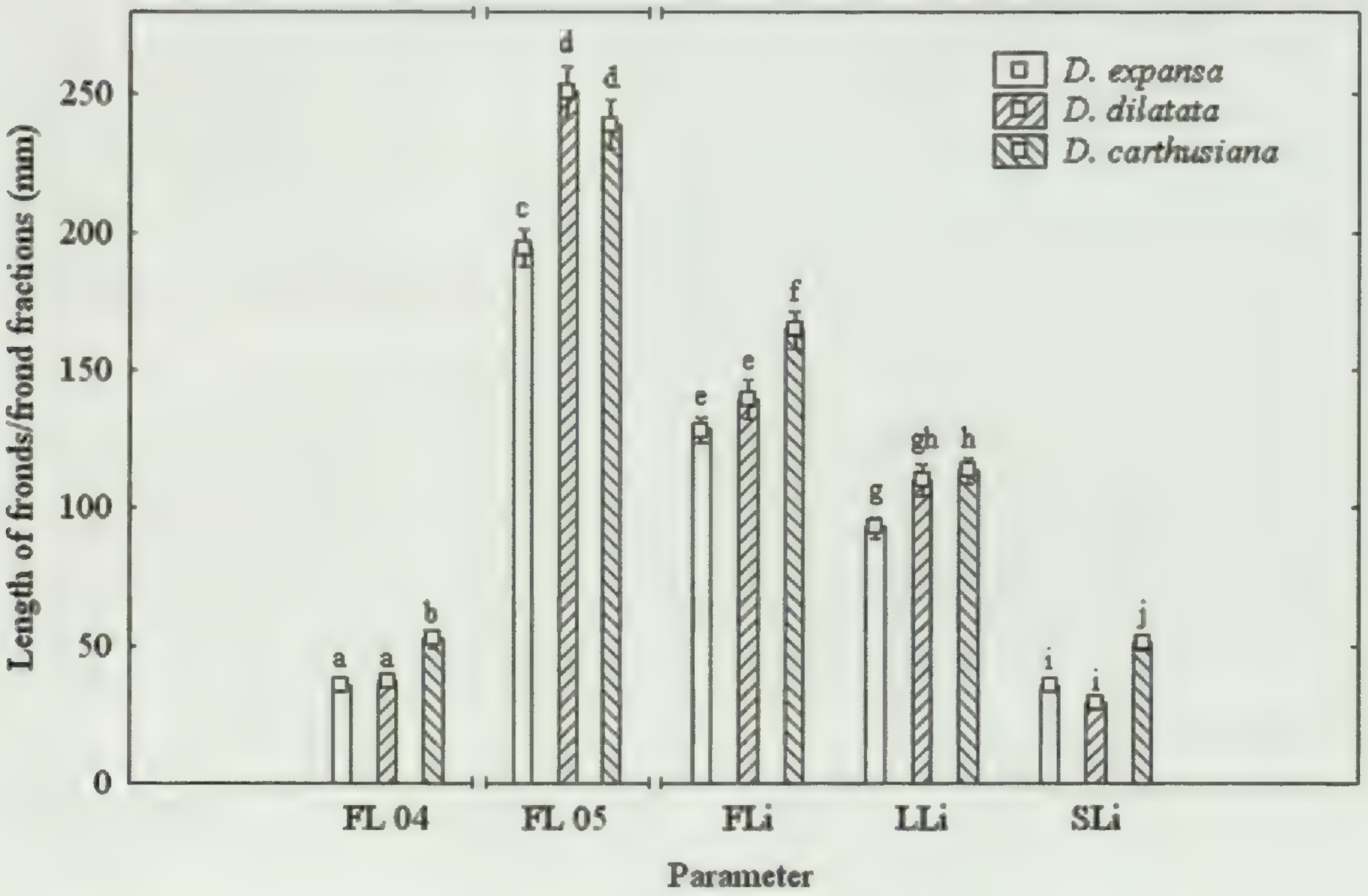

FIG. 2. Mean ± SE of length of fronds of *Dryopteris expansa*, *D. dilatata* and *D. carthusiana*: length of the longest frond in October 2004 (FL 04) and length of the longest frond (FL 05) at the final harvest; length of the fronds (FLi), length of the lamina (LLi) and length of the stipe (SLi) per fern individual (mm) at the final harvest. Bars with the same letter are not significantly different (P < 0.05, Tukey test). X-axis breaks between the results of different analysis.

(total, frond, rhizome and root) and also larger lamina area compared to *D. expansa*. In the case of rhizome mass, the difference between *D. expansa* and *D. dilatata* was marginally non-significant (P = 0.09). There were no differences in SLA between species. The relative biomass allocation pattern was different between species; *D. expansa* allocated significantly more biomass into the rhizome and less into the laminae than *D. dilatata* and *D. carthusiana* (Fig. 4).

Reproductive Traits

Dryopteris dilatata had the lowest proportion of fertile individuals in the final harvest (80.0%), whereas *D. expansa* and *D. carthusiana* had more (93.3% and 86.7% respectively). *Dryopteris dilatata* had significantly fewer fertile fronds compared to the number of its own sterile fronds (t-test: t = 3.178, Df = 11, P = 0.01) and fewer fertile fronds per fertile individual than *D. carthusiana* at the end of the experiment (Fig. 3). *Dryopteris dilatata* also had a smaller pinnae area covered with sori per fertile individual at the final harvest compared to *D. carthusiana* and *D. expansa* (Fig. 5). There was no significant difference between the number of fertile and sterile fronds between the other two species. In the case of *D. carthusiana* and *D. dilatata*, vegetative reproduction was also observed; *D.*

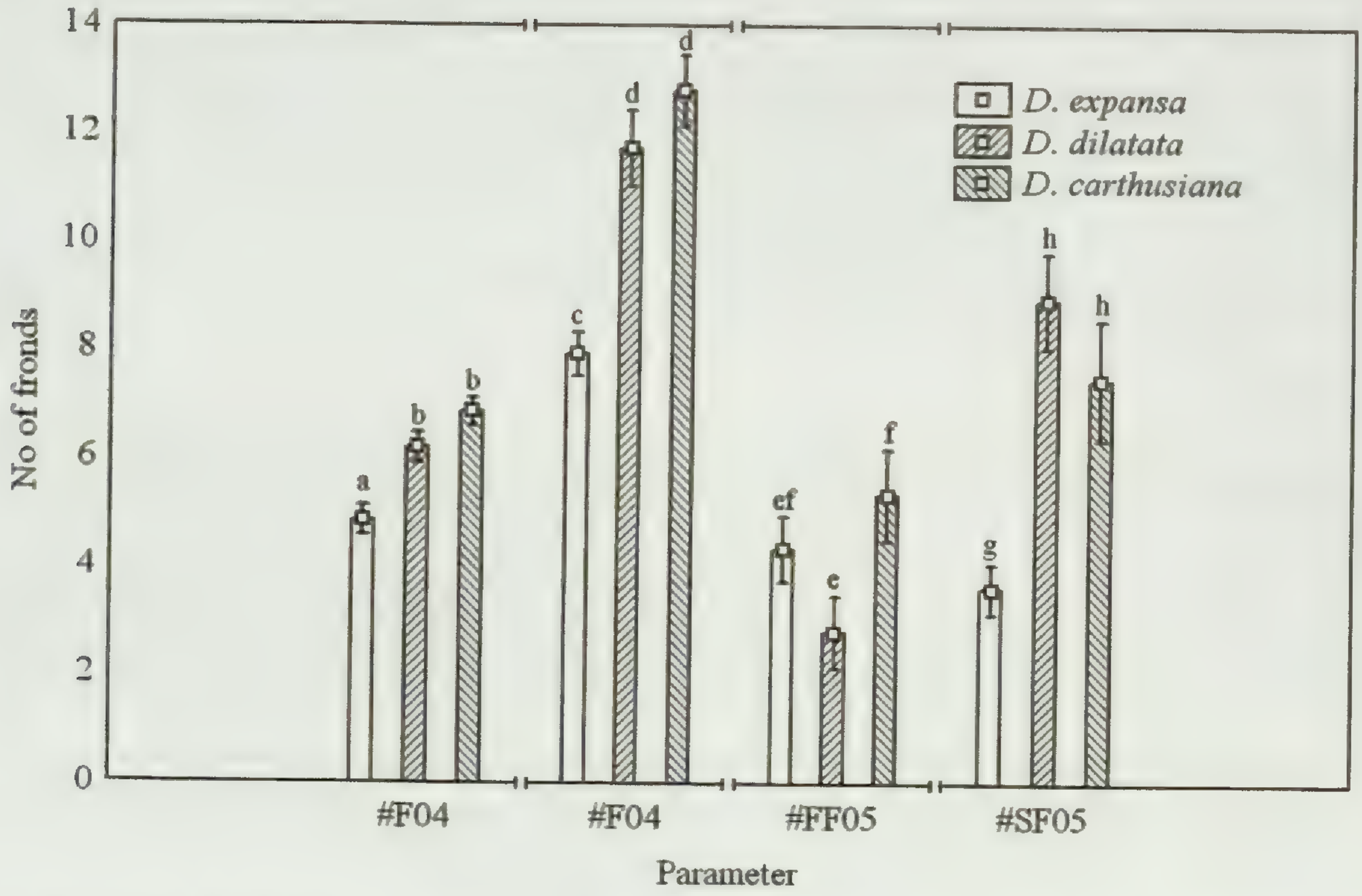

FIG. 3. Mean ± SE of number fronds of *Dryopteris expansa*, *D. dilatata* and *D. carthusiana*: number of fronds per fern individual in October 2004 (#F04), number of fronds (#F05) and number of sterile fronds (#SF 05) per fern individual at the final harvest; number of fertile fronds (#FF05) per generative individual at the final harvest. Bars with the same letter are not significantly different ($p < 0.05$, Tukey test). X-axis breaks between the results of different analysis.

carthusiana had an average of 1.07 vegetative offspring per plant individual and *D. dilatata* 0.07. There was no difference among the species for the time when the first fertile frond appeared; all appeared in August 2005.

DISCUSSION

During the first growth period all three species showed differences in vegetative growth. Intensive growth of *D. carthusiana* for a longer period of time than the other two species resulted in the tallest plants (the longest fronds) by the end of the first growth period and the longest fronds per fern individual by the second growth period. All morphological and biomass parameters, recorded at the end of the experiment, showed that individuals of *D. carthusiana* were larger than those of *D. expansa*. The most successful post-emergence growth may be the crucial precondition for *D. carthusiana*'s high frequency in natural ecosystems. The first vegetation period of young *D. carthusiana* sporophytes was characterized by the longest period of intensive vegetative growth (from June until September), the highest LER, and as a result probably the largest biomass. Achieving higher fertility or utilizing more resources for reproducing could support the finding that the LER of *D. carthusiana* in 2005 was lower than that of *D. dilatata*.

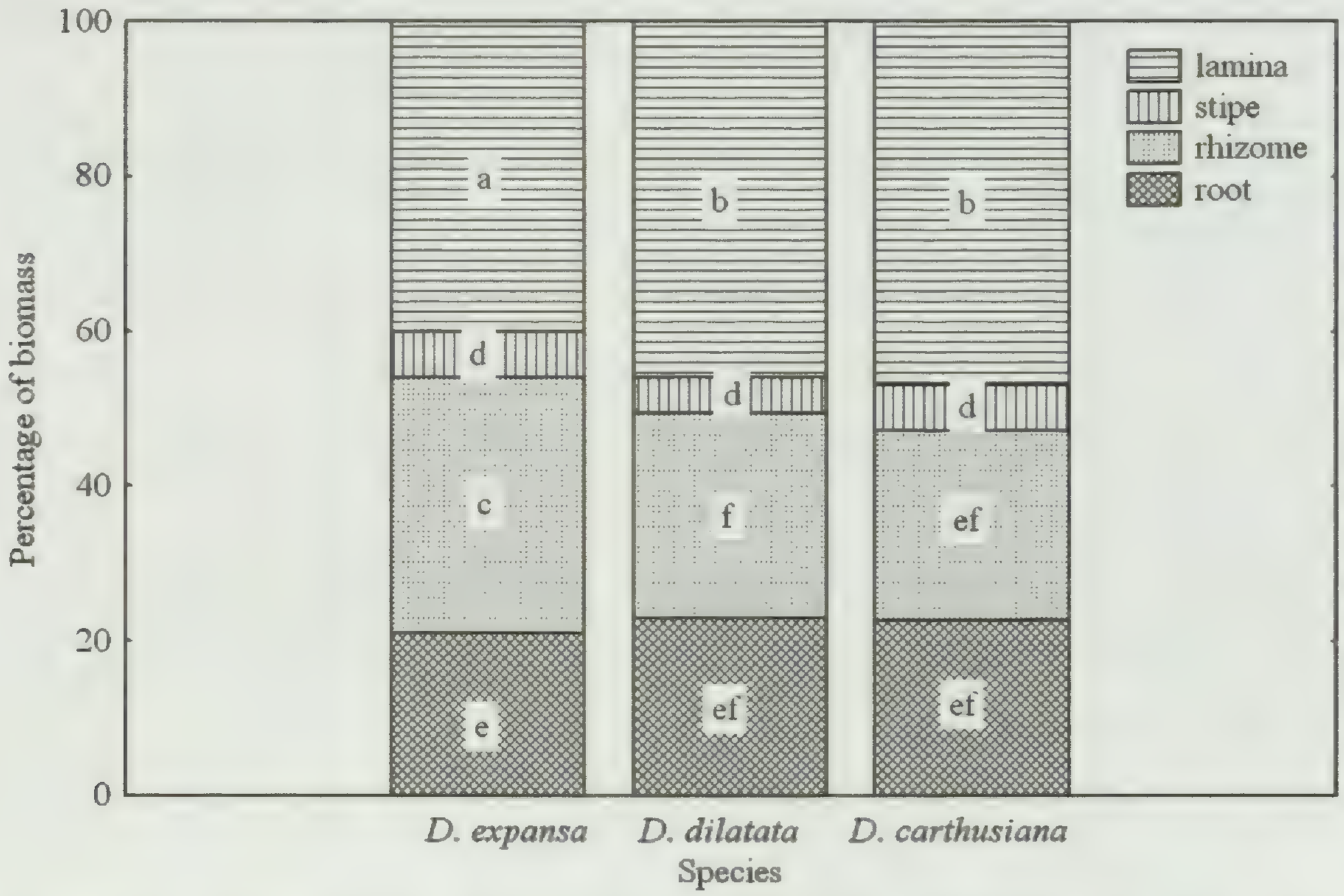

FIG. 4. Mean relative biomass allocation pattern in *Dryopteris carthusiana*, *D. expansa* and *D. dilatata*. Proportions with the same letter are not significantly different ($P < 0.05$, Tukey test).

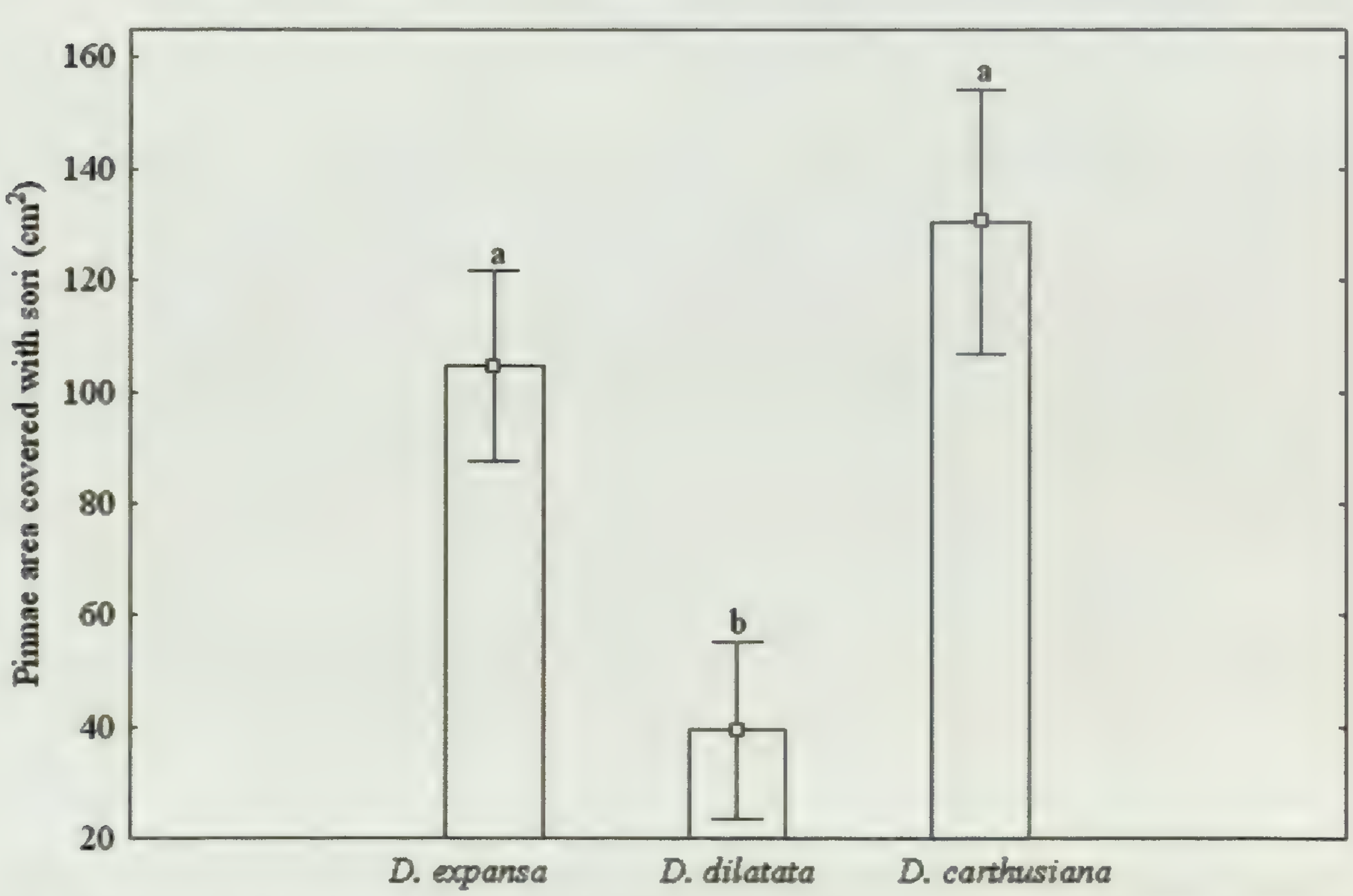

FIG. 5. Mean ± SE of pinnae area covered with sori (cm^2) per generative individual of *Dryopteris expansa*, *D. dilatata* and *D. carthusiana* at the final harvest. Bars with the same letter are not significantly different ($P < 0.05$, Tukey test).

Although none of the reproductive traits of *D. carthusiana* were significantly higher than those of *D. expansa* in the present experiment, the ability of *D. carthusiana* to self-fertilize (in experimental conditions 55% of singly isolated gametophytes grown on soil and even 79% on decomposed wood formed sporophytes; Seifert, 1992) provides the species with a high potential for establishment (Flinn, 2006) and may be an important factor behind its broad distribution. In addition, comparatively high values of vegetative parameters in different light conditions (Rünk and Zobel, 2007), and therefore the high competitive ability (Rünk *et al.*, 2004) may help to explain the highest local (Rünk *et al.*, 2006) and regional frequency (Kukk and Kull, 2005) of *D. carthusiana* among the three species in Estonia.

In the first growth period *D. expansa*, compared to other two species, had the lowest values of frond number parameters (number of fronds in October 2004, increase in number of fronds and NIR in 2004). *Dryopteris expansa*, compared to *D. carthusiana*, had a shorter period of intensive growth, lower LER and lower values of frond growth parameters (length of the longest frond in October 2004 and increase in number of fronds in 2004). The biomass results of the present, two-year experiment related to *D. expansa* were analogous to results of our earlier one-year experiment (Rünk *et al.*, 2004); *D. expansa* had the smallest biomass parameters (total mass, roots mass and frond mass), except in the case of rhizome biomass. We also found a significant difference between *D. expansa* and the other two species in relative biomass allocation, where *D. expansa* invested more biomass in its storage organ, the rhizome, and less in the laminae. The different allocation strategy may be connected with the habitat preferences of this species such as better tolerance to severe climatic factors in mountains or in extreme northern regions of Europe. The relatively short period of intensive vegetative growth (only in June and July) may also have the same explanation.

Although the reproductive success of *D. expansa* in terms of fertile fronds, both in natural (Rünk *et al.*, 2006) and experimental conditions, as well in number of spores, were not lower than of *D. carthusiana*, a low mean intragametophytic selfing rate of 0.34 (Soltis and Soltis, 1987) and thus low establishment ability may have an effect on the distribution frequency of the species. The lower vegetative growth of diploid *D. expansa* and hence lower competitive ability (Rünk *et al.*, 2004) and lower post-emergence growth compared to tetraploid *D. carthusiana* could be connected to the diploid origin and mating system (comparatively low intragametophytic selfing rate) of the species. The differences between diploid and tetraploid species may partly be based on higher levels of inbreeding depression in the case of diploid species (Masuyama and Watano, 1990). Tetraploid fern species are generally larger (Page, 2002), due to heterosis, and have higher rates of spore germination and faster growth rates (Kott and Peterson, 1974).

Considering that *D. dilatata* is a tetraploid, its potential growth ability should be as high as *D. carthusiana*. Still, according to the results of the present experiment, *D. dilatata* had slower leaf elongation rates of young sporophytes during the first growth period, specifically in July and August,

which resulted in shorter plants by the end of September. *Dryopteris dilatata*, compared to *D. expansa*, had taller and a faster increasing number of fronds. *Dryopteris dilatata* had a different growth strategy compared to the other two species. Growth of the longest frond of *D. dilatata* was intensive for only a very short time, in June during the first growth period, similar to *D. expansa*. By contrast *D. dilatata* had an intensive increase in the number of fronds during almost the whole growth period until October, an even longer duration than *D. carthusiana*. The ability of *D. dilatata* to maintain intensive vegetative growth of the longest frond for a longer time may be restricted by some climatic factor. The notable difference in the timing of these two parameters may be connected with the different type of parameters under discussion. Since the frond size is more plastic than the number of fronds, an increase in the number of the fronds was preferred by the trade-off between the two parameters. Consequently, that ability of *D. dilatata* to establish in local vegetation very probably depends on some climatic factor. In better weather conditions *D. dilatata* may grow larger than *D. expansa* in the first growth period (Rünk *et al.*, 2004) and have better post-emergence growth ability. The growth of the species may be slower in less ideal conditions, as in the first growth period and continued in the second of the present experiment. In the second growth period *D. dilatata* achieved the highest LER, had a larger biomass, more and longer fronds than *D. expansa*; however this may occur too late for the successful establishment of the specific cohort and as well for the species.

With regards to the reproductive parameters, *D. dilatata* had the lowest number of spores, the lowest number of fertile individuals and a lower relative number of fertile fronds, compared to the other species. The number of fertile fronds per fertile individual of *D. dilatata* was also the lowest among the three species, although the difference with *D. expansa* was not significant. Taken all together, those differences indicate that in given conditions, the reproductive success of *D. dilatata* might be the lowest. Not only may the unstable establishment abilities limit the distribution of *D. dilatata*, but also its comparatively low self-fertilization rate (only 19.2% gametophytes on soil and 35.2% on decomposed wood produced sporophytes; Seifert, 1992). Therefore a low establishment potential may contribute to the low frequency of this species in Estonia.

In conclusion, the relative population density of the three *Dryopteris* species is related to the relative establishment abilities of the species. *Dryopteris carthusiana* had the highest values of the length parameters of vegetative growth and growth rate in the first growth period and has the highest local population density, while *D. dilatata* and *D. expansa*, both with shorter fronds, shorter intensive growth periods and lower leaf elongation rates, have lower population densities.

Although the short time period of our observatory studies did not allow for any assessment of the dynamics of the distribution of *D. dilatata* in the region, the dynamic population structure (Rünk *et al.*, 2006) and high plasticity (Rünk and Zobel, 2007) of the species might indicate that those species have a good perspective to expand their distribution in the future.

Data made available in 2003 (Blamey *et al.*) has already shown expansion of the distribution of *D. dilatata* in Great Britain and Ireland during the last 40 years. Explanations for the distribution expansion may be the relatively young age of *D. dilatata* (allotetraploid, originated from *D. expansa* and *D. intermedia*), or expansion due to climate warming as already predicted (Bakkenes *et al.*, 2002).

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The Function of Trichomes of an Amphibious Fern, *Marsilea quadrifolia*

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ABSTRACT.—*Marsilea quadrifolia*, an amphibious fern, has the ability to develop heterophyllous, aerial and submerged leaves. In contrast to submerged leaves, aerial leaves have trichomes on both surfaces. To examine if the presence of trichomes can reflect excess light and hence reduce the risk of being damaged by excess light, we compared the optical properties and chlorophyll a fluorescence, in particular the maximum PSII photochemical efficiency (Fv/Fm), of *M. quadrifolia* leaves with trichomes (intact) and those having trichomes removed (de-trichomed). Photosynthetic gas exchange measurements were also conducted to quantify transpirational water loss and instantaneous water use efficiency (WUE) of *M. quadrifolia* intact and de-trichomed leaves. The results showed that removal of trichomes neither affected the optical properties in the visible part of the solar spectrum nor the midday depression of Fv/Fm values of leaflets. In contrast, significantly increased in transpiration rates and decreased rates in WUE were found in de-trichomed leaflets in comparison to intact ones. These results imply that the presence of trichomes is of more importance in reducing water loss than in reflecting light and protecting *M. quadrifolia* against the potentially damaging effect of photoinhibition in aerial environments.

KEY WORDS.—amphibious fern, gas exchange, *Marsilea quadrifolia*, optical property, photoinhibition, trichome

How amphibious species cope with contrasting environmental conditions between aquatic and terrestrial habitats is of interests to researchers. *Marsilea*, an amphibious fern genus, has the ability to develop heterophyll. *Marsilea quadrifolia* L. experiencing extreme variation in environment develops submerged, floating, emergent and terrestrial leaves (Liu, 1984; Lin *et al.*, 2007). These different forms of leaves have different morphological and physiological characteristics. For example, in contrast to the glabrous surface of submerged and floating leaves, the adaxial and abaxial surfaces of emergent leaves are covered with dense trichomes. In addition, leaves of terrestrial grown *M. quadrifolia* (terrestrial leaves) have more trichomes than emergent leaves of aquatic grown (Lin *et al.*, 2007). The ecological importance of these trichomes has not been studied.

The two commonly cited functions of trichomes are to increase reflectance and to increase boundary layer resistance (Lambers *et al.*, 1998). Increasing

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reflectance would reduce incident light on leaf surfaces and might reduce the risk of overheating and the potentially harmful effects of excessive light on leaves (Ehleringer, 1984). Increasing boundary layer resistance would reduce transpirational water loss. A combination of drought, temperature and light stress would greatly increase in terrestrial environments in comparison to that in aquatic habitats. Hence, the production of trichomes may represent one of the acclimation responses conferring *M. quadrifolia* the ability to grow in terrestrial conditions (Lin *et al.*, 2007).

The phenomenon of photoinhibition occurs when leaves are exposed to light levels in excess of what can be utilized in photosynthesis (Powles, 1984). Photoinhibition leads to decreases in photon yield and photosystem II is considered the primary site of photoinhibition (Barber and Andersson, 1992). The yield and kinetics of chlorophyll a fluorescence emitted from leaves upon illumination with actinic light have been used as a probe for the primary photochemistry of photosynthesis (Krause and Weis, 1991). In particular, the linear relationship between quantum yield and the ratio of variable fluorescence to maximum fluorescence (Fv/Fm) (Kao and Forseth, 1992), suggests that Fv/Fm can be used as a probe to monitor the activity of photosynthetic carbon assimilation. A decrease in the values indicates reduction in photosynthetic activity.

The objective of this study is to investigate the ecological significance of trichomes in leaves of *M. quadrifolia*. To examine if the presence of trichomes can reflect excess light and hence reduce the risk of being damaged by excess light, we compared the optical properties and chlorophyll a fluorescence of *M. quadrifolia* intact leaves (with trichomes) with leaves having trichomes removed (by de-trichomed treatment). Photosynthetic gas exchange measurements were also conducted to quantify transpirational water loss and instantaneous water use efficiency (WUE) of *M. quadrifolia* intact and de-trichomed leaves. We test the hypothesis that to reduce the risk of drought stress and being damaged by excessive light, *M. quadrifolia* develops trichomes, reducing transpirational water loss and/or reflecting excess light.

Material and Methods

Rhizomes of *M. quadrifolia* were planted in 2 L plastic pots filled with a mixture of vermiculite: soil of 1:1 by volume. Plants were grown in a glasshouse receiving natural daylight, watered to soil saturation every other day, and fertilized using inorganic fertilizer (N:P:K of 20:20:20) once every two weeks. The plant produces leaves with four leaflets expanded on a plane perpendicular to the petiole, resembling a four-leaf clover, which is connected to the rhizome. The following measurements were conducted on leaflets.

The morphology of trichomes was observed and the length measured under a scanning electron microscope (TM 1000, Hitachi). The optical properties were measured on the same leaflet before and after partial trichomes being removed. To remove trichomes, we gently brushed both surfaces of leaflets. Trichome density was estimated on both surfaces with a dissecting microscope

before and after the de-trichomed treatment. Leaf optical measurements on adaxial surfaces were made using a custom-built dual integrating sphere system following the method described by Runcie and Durako (2004). Briefly, leaf spectral transmittance ($T(\lambda)$) and reflectance ($R(\lambda)$) were measured from 400 nm to 700 nm at 0.5 nm resolution using a fiber-optic spectrometer (HR2000, Ocean Optics) interfaced with a FOIS-1 (for $T(\lambda)$ measurement; Ocean Optics) or ISP-REF (for $R(\lambda)$ measurement; Ocean Optics) integrating spheres. Light source provided by a collimated beam from a tungsten-halogen light (LS-1, Ocean Optics) was directed into the entrance port and to an exit port of the opposite side of the sphere through an optical fiber. For measuring reflectance, measurements were calibrated against a 99% reflectance standard (WS-1-SS, Ocean Optics). After measurements of $T(\lambda)$ and $R(\lambda)$, we then calculated leaf absorptance ($A(\lambda) = 1 - T(\lambda) - R(\lambda)$).

To evaluate if the presence of trichomes can reduce photoinhibition, we measured the characteristics of fluorescence induction on intact and treated leaflets in situ using a portable, pulse amplitude modulated fluorometer (Mini-PAM, Walz, Effeltrich, Germany). Fluorescence was measured on the adaxial side of leaflets with or without trichomes removed ($n = 6$) at 10, 12 and 14 h on a clear day. Leaves were adapted to darkness for 30 minutes before the measurement was taken. Photosynthetic photon flux (PPF) on a horizontal surface at the same height of the leaves and air temperature were also monitored.

Photosaturated photosynthetic rates (A_{max}) and transpiration rates (E) were measured with an LI-6400 infrared gas exchange system (LI-Cor, Lincoln, Nebraska, USA) on the most recently expanded, intact and de-trichomed leaflets. The intercellular CO_2 concentration (Ci) was calculated according to Farquhar and Sharkey (1982). Measurement conditions within the cuvette were: photosynthetic photon flux density (PPF) of 1200 μmol m^{-2} s^{-1}, cuvette temperature 30°C, leaf-to-air water vapor pressure difference (VPD) 1.5–1.6 kPa, and ambient CO_2 concentration 360 ± 5 cm^3 m^{-3}. The de-trichomed leaflets remained green and looked healthy a few days after the experiment indicating that the brushing treatment did not damage the leaflets. To further make sure that the de-trichomed treatment did not damage the epidermis, we also made paraffin sections of leaflets and found that the epidermis cells remained intact after the detachment of trichomes (data not shown).

All statistical tests were performed with the computer software SYSTAT (Statistical Solution, Cork, Ireland). Significant differences are reported as $P < 0.05$.

Results

Characteristics of trichomes.—The morphology of trichomes is shown in the SEM picture (Fig. 1). Grown in terrestrial condition, *M. quadrifolia* produced multicellular, 2–3 cells, trichomes (Fig. 1). Before making the SEM scan, we used liquid nitrogen to fix the samples. Some of trichomes were detached from the surface by the treatment. Hence, we estimated trichome density with a

FIG. 1. Adaxial surface of *M. quadrifolia* leaflet with trichomes.

dissecting microscope instead of calculating the number of trichomes by SEM scanning. The result showed that there was no significant difference in trichome density between the adaxial and abaxial surfaces (Table 1).

Leaf optical properties.—In general, intact and de-trichomed leaflets had the highest reflectance and transmittance and the lowest absorptance at ca. 550 nm in the wavelengths ranging from 400 to 700 nm (Fig. 2). In a comparison of intact and de-trichomed leaves, no significant difference was found either in $T(\lambda)$ or in $R(\lambda)$. Consequently, both types of leaves had similar $A(\lambda)$. As a result,

TABLE 1. Trichome density (cm^{-2}) on adaxial and abaxial surfaces of intact and de-trichomed leaflets and the optical properties of *M. quadrifolia* (mean ± S.E., n = 5). Values within the same row followed by different superscripts represent significant difference at P = 0.05.

Parameters		Intact leaflets	De-trichomed
Trichome density	Adaxial surface	1433 ± 69[a]	149 ± 47[b]
	Abaxial surface	1594 ± 99[a]	159 ± 39[b]
	Total	3027 ± 164[a]	308 ± 77[b]
Optical properties	Reflectance (%)	5.5 ± 0.4[a]	5.3 ± 1.0[a]
	Transmittance (%)	6.5 ± 0.9[a]	7.8 ± 1.1[a]
	Absorptance (%)	88.0 ± 1.0[b]	87.0 ± 1.7[b]

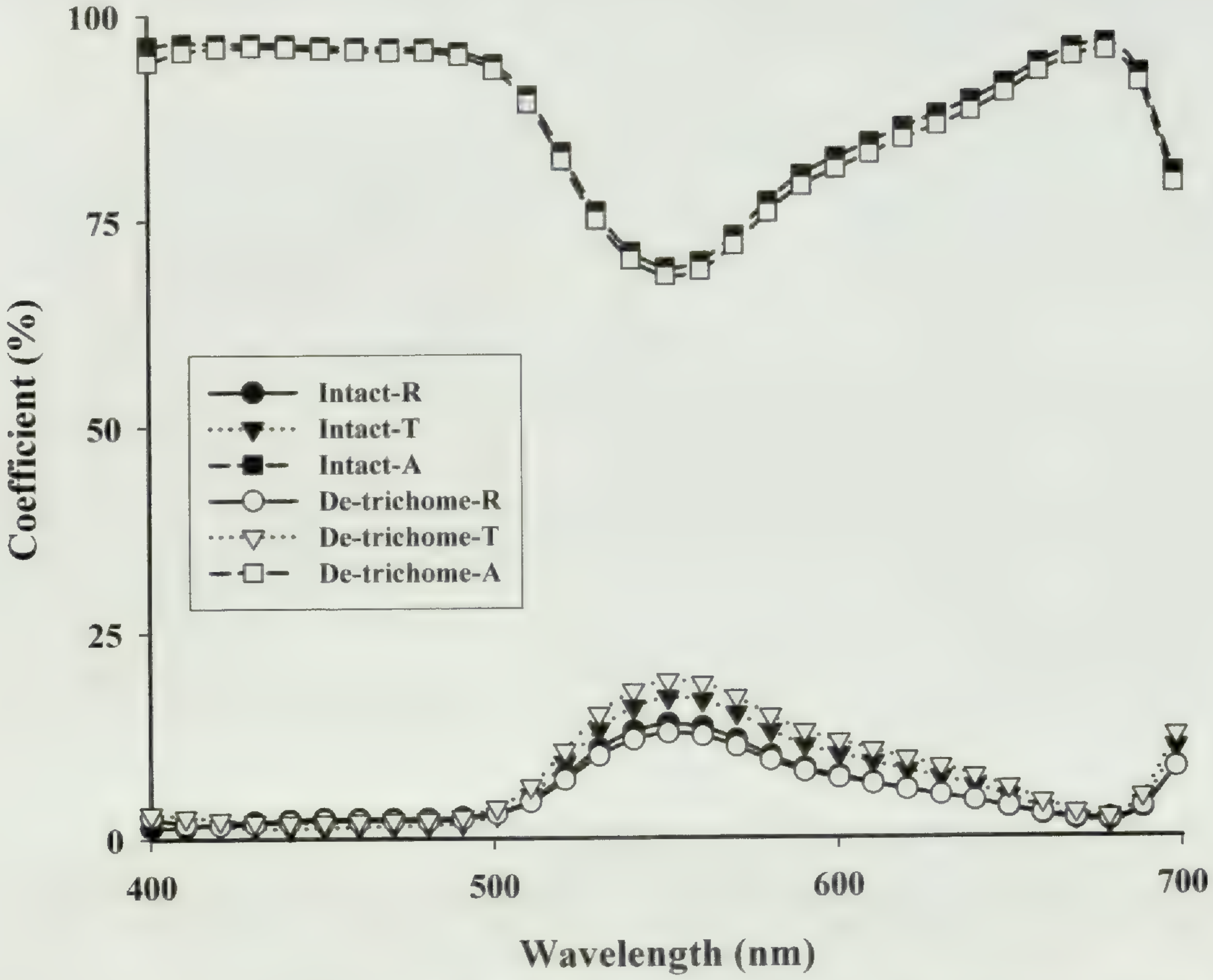

FIG. 2. Average (n = 5) spectral reflectance (R), transmittance (T) and absorptance (A) of *M. quadrifolia* leaftlets before (Intact) and after the removal of tichomes (De-trichomed).

reducing trichome density did not affect the average reflectance, transmittance and absorptance of visible light in *M. quadrifolia* leaflets (Table 1).

Chlorophyll fluorescence measurement.—The diurnal courses of PPF at plant height and air temperature (Ta) were recorded on the same day as leaf fluorescence was measured (Fig. 3a). Initial and continuous dark adapted measurements of maximum PSII photochemical efficiency (Fv/Fm) indicated that the leaves were healthy and not experiencing stress due to the removal of trichomes (Table 2). Midday depression of Fv/Fm values was found in the leaflets when exposed to solar irradiation (Fig. 3b). In comparison to continuously dark-adapted leaves, illuminated leaflets showed significant reduction in Fv/Fm at 1000h, 1200h and 1400h when PPF and air temperature were highest during the day (Fig. 3). No significant difference was found in Fv/Fm values between intact and de-trichomed leaflets.

Gas exchange measurement.—Transpiration rate (E) increases with A_{max} in both intact and detrichomed leaflets; as a result, a significant, positive, linear relationship was found between A_{max} and E (Fig. 4a). No significant difference was found between the slopes of these positive relationships for intact and de-

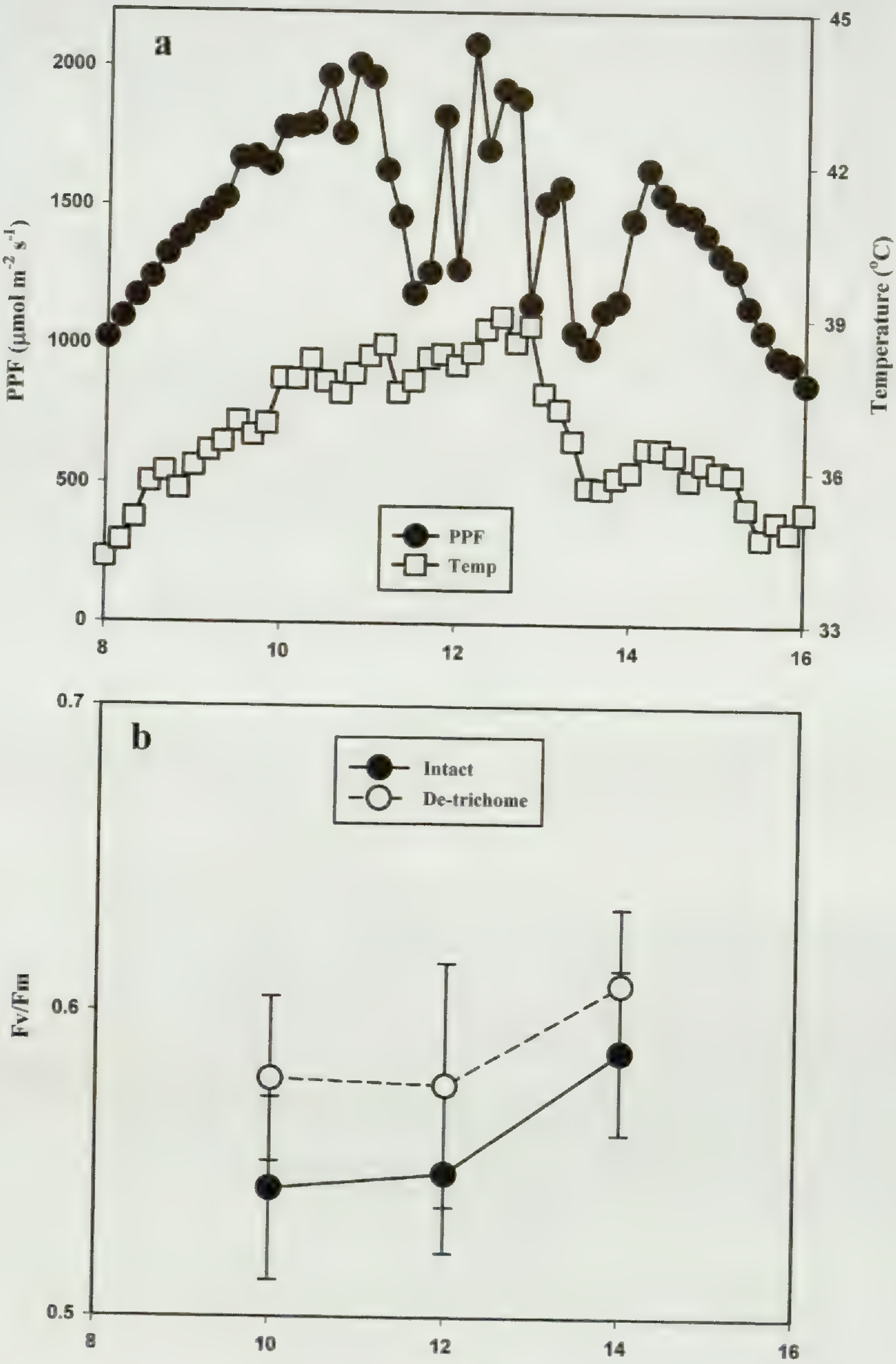

FIG. 3. Diurnal changes in air temperature and PPF incidence on a horizontal surface (a) and the ratio of Fv to Fm (mean ± s. e., n = 6) measured at 1000, 1200 and 1400 h on naturally orienting leaflets of *M. quadrifolia* with (De-trichomed) and without (Intact) removal of trichomes.

TABLE 2. Maximum PSII photochemical efficiency (Fv/Fm) of initial (before leaflet being exposed to solar irradiation) and continuous dark adapted intact and de-trichomed leaflets of *M. quadrifolia* used in the measurement of chlorophyll fluorescence (mean ± s.e., n = 6).

Treatments	Initial	Continuous dark
Intact	0.76 ± 0.01	0.77 ± 0.01
De-trichomed	0.77 ± 0.01	0.77 ± 0.01

trichomed leaflets. However, de-trichomed leaflets had significantly higher E relative to intact leaflets for a given A_{max}.

The calculated Ci values of de-trichomed leaflets were higher than those of intact leaflets (Fig. 4b).

DISCUSSION

Knowledge of how fern species cope with excess light or drought stress in terrestrial environments is of ecological and evolutionary importance. Biochemical mechanisms, such as xanthophyll-mediated energy dissipation, and/or morphological mechanisms, such as leaf curing and laminar scales, have been demonstrated in pteridophytes (Eichmeier *et al.*, 1993; Tausz *et al.*, 2001; Watkins *et al.*, 2006). To our knowledge, the influence of pubescence on the incident radiation and water budget has not been studied in any fern species.

Among other functions (Johnson, 1975; Zvereva *et al.*, 1998), pubescence had been shown to increase reflectance (Ehleringer, 1984; Holmes and Keiller, 2002) and afford protection against excess radiation (Ripley *et al.*, 1999; Morales *et al.*, 2002; Manetas, 2003) in seed plants. Our results, however, showed that *M. quadrifolia* leaflets have a very high absorptance and de-trichomed treatments did not affect the visible part (400–700 nm) of optical properties of the leaflets (Fig. 2). Additionally, the Fv/Fm values of leaflets of *M. quadrifolia* at the midday were not affected by the removal of trichomes (Fig. 3b). It is therefore possible that trichomes on leaflets of *M. quadrifolia* are of less importance in reflecting light and in protecting the plant against the potentially damaging effect of photoinhibition. However, leaf temperatures in de-trichomed leaflets may be reduced due to their increased transpiration rates (Fig. 4a), which may ameliorate the damaging effect of high light and high air temperature on de-trichomed leaflets. For example, the result that the de-trichomed/orienting leaflets had less reduction in Fv/Fm, though not significant, than intact/orienting leaflets (Fig. 3b) might result from the increased transpiration rate in the former. Accordingly, we cannot completely exclude the role of trichomes in providing photoprotection for *M. quadrifolia* leaflets.

Few studies have also shown that the presence of trichomes can reduce leaf transpiration rates (Ripley *et al.*, 1999). The significantly increase in transpiration rates, about 30%, measured in de-trichomed *M. quadrifolia* leaflets compared to intact ones (Fig. 4a) suggests that the presence of

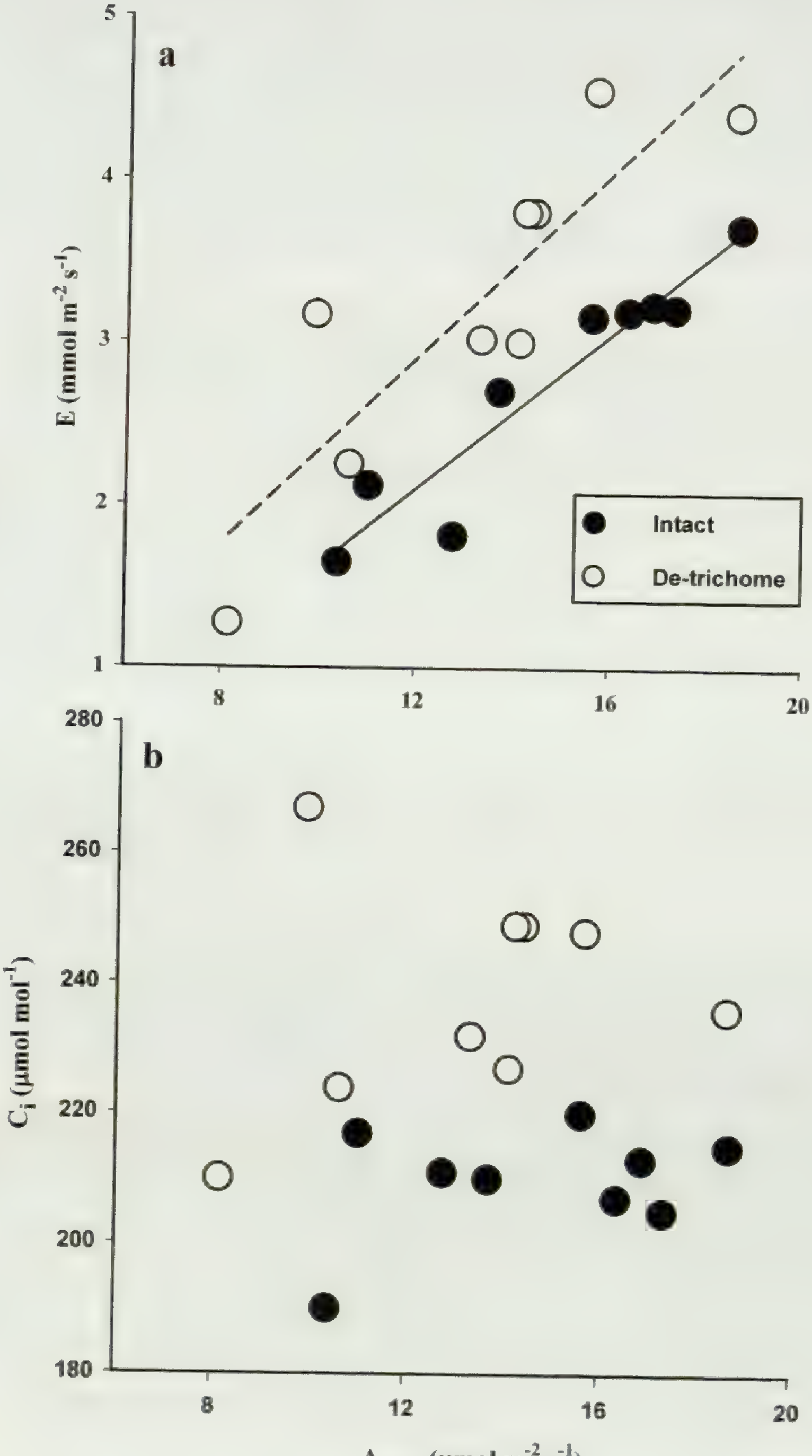

FIG. 4. The relationship between photosaturated photosynthetic rate (A_{max}) and transpiration rates (E) (a) and intercellular CO_2 concentration (Ci) (b) of *M. quadrifolia* leaflets without (Intact) and with removal of trichomes (De-trichomed).

trichomes is of more importance in reducing water loss than in providing photoprotection. At constant leaf-air vapor pressure deficit, Ci values may be used as a measure of the instantaneous water use efficiency (WUE) of the leaf (Farquhar and Sharkey, 1982), with a lower Ci indicating a higher instantaneous WUE. De-trichomed treatments resulted in leaflets with higher Ci (Fig. 4b) implying a lower WUE. These results reveal that the reduction in water loss from *M. quadrifolia* leaflets with trichomes also resulted in increased WUE.

The hair layer on leaves may lead to higher leaf temperatures caused by reducing the transpirational water loss (Ripley *et al.*, 1999). We have observed that leaflets of *M. quadrifolia* have the ability of performing tropic movements (pers. obs.). It is possible that *M. quadrifolia* adjust leaflet angle and azimuth to intercept a smaller quantity of radiant energy, which would allow the plant to moderate leaflet temperature without excessive transpiration. The function of tropic leaf movements in protecting soybean leaves from photoinhibition has been documented (Kao and Forseth, 1992). Thus, the presence of trichomes on both surfaces combined with leaflet movements may provide *M. quadrifolia* mechanisms against drought stress. Accordingly, we hypothesize that the combination of the avoidance mechanisms, leaf movements and the production of trichomes are very important adaptations, conferring the amphibious *M. quadrifolia* ability to grow in terrestrial conditions. The effect of the interaction between water availability and light intensity on trichome density and leaflet movements in *M. quadrifolia* are currently under studied.

Acknowledgments

We thank Dr. Bai-Ling Lin, for the inspiration of this study and providing rhizomes of *M. quadrifolia*, Dr. Shiang-Jiuun Chen for providing technique support in taking the SEM picture, and Yih-Chi Chang, for help in taking chlorophyll fluorescence and gas exchange measurements.

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American Fern Journal 99(4):333–334 (2009)

Shorter Notes

***Isoetes duriei* New to Lebanon**.—In a recent paper, we (Bolin *et al.*, Turkish Journal of Botany 32:447–457. 2008) discussed the taxonomy and distribution of the quillworts (species of the genus *Isoetes*, Lycophyta) in Western Asia. In this supplementary note, we record the presence of three quillworts new to Lebanon –one a widespread Mediterranean species, one known from only a single site in Turkey and two in Syria, and an undescribed new species. With this report, the number of documented species in Lebanon has increased from one to three. Voucher specimens will be deposited at BEI, E, and ODU.

In his flora, Mouterde (*Nouvelle Flora du Liban et de la Syria*. Beirut: Editions de L'Imprimerie Catholique. 1966) included two species of *Isoetes* from Syria and Lebanon– *Isoetes olympica* A. Braun known from only a few sites on Jebel Al Arab (historically known as Jebel Druze) in extreme southeastern Syria, and what Mouterde called *I. histrix* Bory forma *subinermis* Durieu from the Akkar region of northern Lebanon. He separated the two species chiefly on the basis of velum coverage—*I. olympica* with an incomplete velum and *I. histrix* forma *subinermis* has complete velum coverage. Musselman (*Fern Gaz.* 16(6, 7 & 8):324–3 29. 2002) noted the impending demise of the Jebel Al Arab populations due to habitat destruction. However, in 2007 populations of *I. olympica* were found at several sites in the vicinity of Homs, Syria (Bolin *et al.*, 2008).

In April 2009, we located thousands of quillworts at several different sites in the Akkar region of extreme northern Lebanon, a region of basalt derived soils. Examination of the megaspores showed clearly that they are *I. duriei* Bory, a Mediterranean species previously unknown in the eastern Mediterranean with the closest populations in Turkey (Bolin *et al.*, 2008). Unlike most species of quillworts, *I. duriei* is terrestrial and grows in typical garrigue (degenerated Mediterranean forest) vegetation. Plants were small and initially difficult to locate among the grasses and forbs. Based on the large number of plants we saw, it is hard to understand how this plant could have been overlooked after more than a century and a half of botanical studies in Lebanon. This may be due to their maturation as early as mid-April, plants were beginning to senesce and had mature spores at this stage. The large megaspores of *I. duriei* with distinctive alveolate ornamentation are easy to recognize, being among the most distinctive in the genus.

Near the village of Kfar Noun, especially robust plants of *I. olympica*, readily discerned by the incomplete velum and much smaller tuberculate megaspores, were abundant in a vineyard among numerous *I. duriei*. *Isoetes olympica* has never been reported from Lebanon. For almost a century, *I. olympica* was known only from the type locality near modern day Bursa, Turkey. In the 1930's several populations were found at Jebel Druze (Musselman, 2002). The discovery of large populations near Homs and the recently discovered Akkar plants strongly suggests that *I. olympica* has a much wider distribution and is

more abundant than previously thought. It should be sought at additional sites in Syria, as well as eastern Turkey and Iraq. Images of *I. olympica* and *I. duriei* from Akkar are at: http://www.odu.edu/~lmusselm/plant/index.php

In addition to *I. olympica* and *I. duriei* we found a third species which is apparently new to science. Hybrids are known from most places in the world where two or more species grow together and we have recently noted the first hybrids involving *I. duriei* (with *I. histrix*)(Bolin *et al.*, 2008).

The addition of these species to the flora of Lebanon is significant for two reasons. It is the first report of *I. duriei* in the eastern Mediterranean. We have also documented new populations of *I. olympica* formerly thought to be of great conservation concern. The only quillwort we have not yet found in Lebanon is *I. histrix* forma *subinermis* (sometimes known as *I. subinermis* (Bory) Cesca & Peruzzi, see Bolin *et al.*, 2008). Because this taxon, sensu Mouterde, has a complete velum, it must include *I. duriei* and *I. olympica*. It is likely that additional quillwort species could be found in the eastern Mediterranean and we hope that this note will help botanists be aware of these easily overlooked plants.—Lytton J. Musselman and Mohammad S. Al-Zein, Department of Biological Sciences, Old Dominion University, Norfolk, Virginia 23529-0266, USA.

American Fern Journal 99(4):335 (2009)

Errata

AFJ volume 98, issue 4, pp. 214–250 (October–December 2008)

The following errors have been kindly pointed out by Alan R. Smith, UC Berkeley, in Lehnert, M. (2008). Eleven new species in the grammitid fern genus *Melpomene* (Polypodiaceae). American Fern Journal 98(4): 214–250.

p. 219 – *M. albicans*: *Lehnert 512, 599, 601, 602* not at UC.
p. 219 – *M. albicans*: *Lehnert 707* should be *709*.
p. 221 – *M. caput-gorgonis*: the type collection *Lehnert 367* (p. 219) is cited also as an additional specimen examined.
p. 219 – *M. caput-gorgonis*: isotype *Lehnert 367* not at UC, but LPB.
p. 221 – *M. caput-gorgonis*: *Lehnert 386* not at UC but MO, *Lehnert 392* not at UC.
p. 222 – *M. caput-gorgonis*: *Lehnert 496a, 586* not at UC.
p. 224 – *M. flagellata*: *Jiménez I. & Gallegos 527* must read *725*.
p. 229 – *M. jimenezii*: In the additional specimens examined, the collector *Perea* is misspelled *Perera*.
p. 230 – *M. michaelis*: In order to conserve the writing of the epithet, the dedication of this species is restricted to Michael Sundue. A singular of the name as *pars pro toto* is apparently not tenable.
p. 231 – *M. michaelis*: The type collection *Lehnert 519* (p. 229) is also cited as an additional specimen examined.
p. 234 – *M. occidentalis*: *Lehnert 1464a, 1558a* not at UC.
p. 236 – *M. paradoxa*: the type collection *Kessler et al. 11717* (p. 235) is cited also as an additional specimen examined.
p. 236 – *M. paradoxa*: *Lehnert 536, 542* not at UC.
p. 241 – *M. personata*: *Kessler et al. 6862* must be *6862B*, not at UC.
p. 241 – *M. personata*: *Lehnert 404* not at UC.
p. 244 – *M. sklenarii*: *Sklenar & Sklenarova 2803* not at UC.
p. 249 – *M. vulcanica*: *Lehnert 168, 174* not at UC.

Marcus Lehnert, Staatliches Museum für Naturkunde Stuttgart, Am Löwentor, Rosenstein 1, D-70191 Stuttgart, Germany.

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Referees for 2009

All papers submitted to the journal are peer reviewed. Members of the editorial board and the American Fern Society, as well as additional scientists in cognate areas do these reviews on a voluntary basis. It is their work that contributes to the high quality of articles in the *American Fern Journal* and to its continued success. The American Fern Society and I extend our thanks to the following reviewers for the assistance, diligence, and patience in the year 2009 (I apologize if I inadvertently omitted anyone from this list).

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Publication title and number: *American Fern Journal* (0002-8444). Date of filing: September 26, 2009. Frequency of issue: quarterly. Annual subscription price 2009: $25.00. Office of Publisher: c/o Missouri Botanical Garden, P.O. Box 299, St. Louis, MO 63166-0299. Editor: Jennifer Geiger. *The American Fern Journal* is wholly owned by the American Fern Society, Inc., with no bond holders. Physical address of the Society: c/o Missouri Botanical Garden, 4344 Shaw Blvd., St. Louis, MO 63110. The purpose, function, and nonprofit status of the Society and its tax exempt status for Federal income tax purposes remains the same as in past years. The average press run for Volume 99 is 912, and was 900 for the issue appearing immediately prior to the filing date, for which 760 copies were mailed as paid circulation and 0 copies were mailed as free distribution, leaving 134 copies for office use and back-issues sales. I certify that these statements are correct and complete. George Yatskievych, Membership Secretary of AFS.

Table of Contents for Volume 99
(A list of articles arranged alphabetically by author)

Al-Hamdani, S. H. and J. J. Ghazal. Selected Physiological Responses of *Salvinia minima* to Various Temperatures and Light Intensities 154

Al-Zein, M. S. (see L. J. Musselman) 333

Anderson, O. R. Eukaryotic Microbial Communities Associated with the Rhizosphere of the Temperate Fern *Thelypteris noveboracensis* (L.) Nieuwl. ... 175

Arosa, M. L., L. G. Quintanilla, J. A. Ramos, R. Ceia and H. Sampaio. Spore Maturation and Release of Two Evergreen Macaronesian Ferns, *Culcita macrocarpa* and *Woodwardia radicans*, along an Altitudinal Gradient ... 260

Ayrapetov, A. and M. T. Ganger. Nutrient Levels Do Not Affect Male Gametophyte Induction by Antheridiogen in *Ceratopteris richardii* 273

Barker, M. S. and G. Yatskievych. Summary of the 2008 AFS Symposium: From Gels to Genomics: The Evolving Landscape of Pteridology. A Celebration of Gerald Gastony's Contributions to Fern Evolutionary Biology 117

Barker, M. S. and G. Yatskievych. A Brief History of Gerald J. Gastony's Botanical Career 117

Barker, M. S. Evolutionary Genomic Analyses of Ferns Reveal that High Chromosome Numbers are a Product of High Retention and Fewer Rounds of Polyploidy Relative to Angiosperms 136

Barrington, D. S. (see G. J. Gastony) 231

Beck, J. (see M. D. Windham) 128

Caluff, M. G. A New Species of *Adiantum* from Cuba 93

Cardelús, C. (see J. E. Watkins Jr.) 161

Ceia, R. (see M. L. Arosa) 260

Chandra, S. (see A. Srivastava) 181

Chang, H.-M., W.-L. Chiou and J.-C. Wang. Molecular Evidence for Genetic Heterogeneity and the Hybrid Origin of *Acrorumohra subreflexipinna* from Taiwan 61

Chiou, W.-L. (see H.-M. Chang) 61

Christenhusz, M. J. M. Type Specimens of *Dracoglossum sinuatum* Uncovered in the Rio de Janeiro Herbarium 58

Conant, D. S. (see G. J. Gastony) 231

Dubuisson, J.-Y. (see A. Ebihara) 200

Duffy, A. M. (see P. G. Wolf) 132

EBIHARA, A., J. H. NITTA, D. LORENCE and J.-Y. DUBUISSON. New Records of *Polyphlebium borbonicum*, an African Filmy Fern, in the New World and Polynesia 200

ELEUTÉRIO, A. A. and D. PÉREZ-SALICRUP. Transplanting Tree Ferns to Promote Their Conservation in Mexico 279

FARRAR, D. R. (see P. F. ZIKA) 249

FIORI, C. C. L., M. SANTOS and A. M. RANDI. Aspects of Gametophyte Development of *Dicksonia sellowiana* Hook (Dicksoniaceae): an Endangered Tree Fern Indigenous to South and Central America 207

GANGER, M. T. (see A. AYRAPETOV) 273

GANGULY, G., K. SARKAR, and R. MUKHOPADHYAY. *In vitro* Study on Gametophyte Development of an Epiphytic Fern *Arthromeris himalayensis* (Hook.) Ching of South Sikkim, India 217

GASTONY, G. J., D. S. BARRINGTON, and D. S. CONANT. Obituary: Alice Faber Tryon (1920–2009) 231

GHAZAL, J. J. (see S. H. AL-HAMDANI) 154

GRUSZ, A. L. (see M. D. WINDHAM) 128

GUO, X.-S. and B. LI. On *Neolepisorus emeiensis* and *N. dengii* (Polypodiaceae) from China 244

HAUFLER, C. H. Gels and Genetics: The Historical Impact of Isozymes on Paradigm Shifts in Hypotheses about Fern Evolutionary Biology 125

HICKEY, R. J., C. C. MACLUF and M. LINK-PÉREZ. *Isoetes maxima*, a New Species from Brazil 194

HOSHIZAKI, B. J. Illustrated Flora of Ferns and Fern Allies of South Pacific Islands 59

HSU, R. (see C. E. MARTIN) 145

HUA, W., C. PING-TING, Y. LI-PING and C. LONG-QING. An Efficient Method for Surface Sterilization and Sowing Fern Spores *in vitro* 226

HUIET, L. (see M. D. WINDHAM) 128

KAO, W.-Y. (see T.-C. WU) 323

KESSLER, M. (see M. LEHNERT) 292

KOTTKE, I. (see M. LEHNERT) 292

LABIAK, P. H. (see F. B. MATOS) 101

LABIAK, P. H. (see R. C. MORAN) 1

LEHNERT, M., I. KOTTKE, S. SETARO, L. F. PAZMIÑO, J. P. SUÁREZ, and M. KESSLER. Mycorrhizal Associations in Ferns from Southern Ecuador 292

LI, B. (see X.-S. GUO) 244

LI-PING, Y. (see W. HUA) 226
LIN, T.-C. (see C. E. MARTIN) 145
LINK-PÉREZ, M. (see R. J. HICKEY) 194
LONG-QING. C. (see W. HUA) 226
LORENCE, D. (see A. EBIHARA) 200
MACLUF, C. C. (see R. J. HICKEY) 194
MARTIN, C. E., R. HSU, and T.-C. LIN. Comparative Photosynthetic Capacity of Abaxial and Adaxial Leaf Sides as Related to Exposure in Two Epiphytic Ferns in a Subtropical Rainforest in Northeastern Taiwan 145
MATOS, F. B., P. H. LABIAK, and L. S. SYLVESTRE. A New Brazilian Species of the Genus Asplenium L. (Aspleniaceae) 101
MICKEL, J. T. (see J. D. TEJERO-DÍEZ) 109
MOLINA, M., V. REYES-GARCÍA and M. PARDO-DE-SANTAYANA. Local Knowledge and Management of the Royal Fern (*Osmunda regalis* L.) in Northern Spain: Implications for Biodiversity Conservation 45
MORA-OLIVO, A. and G. YATSKIEVYCH. *Salvinia molesta* in Mexico 56
MORAN, R. C., J. PRADO and P. H. LABIAK. Megalastrum (Dryopteridaceae) in Brazil, Paraguay, and Uruguay 1
MUKHOPADHYAY, R. (see G. GANGULY) 217
MUSSELMAN, L. J. and M. S. AL-ZEIN. *Isoetes duriei* New to Lebanon 333
NAKAZATO, T. Fern Genome Structure and Evolution 134
NITTA, J. H. (see A. EBIHARA) 200
PAZMIÑO, L. F. (see M. LEHNERT) 292
PARDO-DE-SANTAYANA, M. (see M. MOLINA) 45
PÉREZ-SALICRUP, D. (see A. A. ELEUTÉRIO) 279
PING-TING, C. (see W. HUA) 226
PRADO, J. (see R. C. MORAN) 1
PRYER, K. M. (see M. D. WINDHAM) 128
QI, X., Y. YANG, Y. SU, and T. WANG. Molecular Cloning and Sequence Analysis of Cyanovirin-N Homology Gene in *Ceratopteris thalictroides* 78
QUINTANILLA, L. G. (see M. L. AROSA) 260
REYES-GARCÍA, V. (see M. MOLINA) 45
RAIMONDO, F. M. (see A. TROIA) 238
RAMOS, J. A. (see M. L. AROSA) 260

RANDI, A. M. (see C. C. L. FIORI) 207

ROPER, J. M. (see P. G. WOLF) 132

ROTHFELS, C. (see M. D. WINDHAM) 128

RÜNK, K. and M. ZOBEL. Differences In Post-Emergence Growth Of Three Fern Species Could Help Explain Their Varying Local Abundance 307

SALINO, A. New Combinations in *Pleopeltis* (Polypodiaceae) from Southeastern Brazil 106

SAMPAIO, H. (see M. L. AROSA) 260

SANTOS, M. (see C. C. L. FIORI) 207

SARKAR, K. (see G. GANGULY) 217

SCHUETTPELZ, E. (see M. D. WINDHAM) 128

SETARO, S. (see M. LEHNERT) 292

SHANKAR, R. and R. C. SRIVASTAVA. Obituary: Prem Kumar Khare (1946–2009) ... 236

SMITH, A. R. (see J. D. TEJERO-DÍEZ) 109

SMITH, A. R. Flora de Nicaragua. Tomo 4. Helechos 142

SRIVASTAVA, A. and S. CHANDRA. Structure and Organization of the Rhizome Vascular System of Four *Polypodium* Species 181

SRIVASTAVA, R. C. (see R. SHANKAR) 236

SU, Y. (see X. QI) 78

SUÁREZ, J. P. (see M. LEHNERT) 292

SYLVESTRE, L. S. (see F. B. MATOS) 101

TEJERO-DÍEZ, J. D., J. T. MICKEL, and A. R. SMITH. A Hybrid *Phlebodium* (Polypodiaceae, Polypodiophyta) and Its Influence on the Circumscription of the Genus 109

TROIA, A. and F. M. RAIMONDO. *Isoëtes todaroana* (Isoëtaceae, Lycopodiophyta), a New Species from Sicily (Italy) 238

WANG, J.-C. (see H.-M. CHANG) 61

WANG, T. (see X. QI) 78

WATKINS, J. E. Jr. and C. CARDELÚS. Habitat Differentiation of Ferns in a Lowland Tropical Rain Forest. 161

WINDHAM, M. D., L. HUIET, E. SCHUETTPELZ, A. L. GRUSZ, C. ROTHFELS, J. BECK, G. YATSKIEVYCH, and K. M. PRYER. Using Plastid and Nuclear DNA Sequences to Redraw Generic Boundaries and Demystify Species Complexes in Cheilanthoid Ferns 128

WOLF, P. G., A. M. DUFFY, and J. M. ROPER. Phylogenetic Use of Inversions in Fern Chloroplast Genomes 132

Wu, T.-C. and W.-Y. Kao. The Function of Trichomes of an Amphibious Fern, *Marsilea quadrifolia* 323

Yang, Y. (see X. Qi) 78

Yatskievych, G. (see A. Mora-Olivo) 56

Yatskievych, G. (see M. S. Barker) 117

Yatskievych, G. (see M. D. Windham) 128

Zika, P. F. and D. R. Farrar. *Botrychium ascendens* W. H. Wagner (Ophioglossaceae) in Newfoundland and Notes on its Origin 249

Zobel, M. (see K. Rünk) 307

Volume 99, number 1, January–March, pages 1–60, issued 1 June 2009

Volume 99, number 2, April–June, pages 61–144, issued 1 September 2009

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INFORMATION FOR AUTHORS

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